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PROGRESS

VARIATIONS IN SERUM MAGNESIUM IN HEALTH AND DISEASE: A REVIEW*

VICTOR G. HAURY, M.D., PHILADELPHIA, PA.

INTRODUCTION

UNTIL 1932, when Kruse and his co-workers[1] demonstrated that magnesium is essential for the maintenance of life, very little physiologic significance was attached to it. In recent years, however, much more attention has been given to this element, particularly in regard to its variation in diseased states. It is unfortunate that such abundant researches on the biological significance of calcium should have been performed with an almost total omission of concomitant magnesium studies. This is all the more unfortunate since we now appreciate the close physiologic relationship which exists between calcium and magnesium.

The literature abounds with evidence that a derangement in calcium metabolism is either the cause or the effect of various clinical entities. Such information could have been obtained only by virtue of a great many studies made by the clinical chemists. Obviously the same must be done before any clinical significance can be attached to magnesium. A quotation from a recent editorial on "Magnesium in Nutrition"[2] adequately summarizes the present status of the magnesium problem. . . . "So little is known of the function of magnesium in the organism that clinically observable abnormalities in man cannot at present be said with certainty to be due to magnesium deficiency or to a disorder of magnesium metabolism. The systematic study of magnesium metabolism by accurate analytical and experimental methods is little more than begun. Future investigations may be expected to add considerably to our knowledge of this problem."

*From the Department of Pharmacology, the Jefferson Medical College of Philadelphia.
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Our attempt in this paper has been to review and to evaluate the scattered literature relative to the changes in blood magnesium in various diseases. The material is arranged, in so far as is possible, under the various clinical entities.

No definite conclusions can be drawn in most instances as to whether or not a hypomagnesemia or hypermagnesemia exists in a particular disease. Indeed, in some instances diametrically opposite results are reported by the various investigators. We can only report the facts as they are found, make criticisms where they are warranted, and point out certain trends if sufficient data existed to do so. It is hoped that this paper may serve to correlate, at least to some degree, the literature on this subject, and to stimulate further investigations on this vital and interesting subject.

NORMAL BLOOD VALUES

There is some disagreement in the literature as to what should be considered the serum magnesium level of normal persons. This discrepancy is due partly to the fact that the investigators used different methods for the determination of this element, and partly because some[19] considered results obtained from hospital patients as being normal. Obviously, such persons should not be considered as being normal, even if they do not have any apparent metabolic disturbances. The concentration of normal serum magnesium as determined by 18 different authors and the methods employed by each are shown in Table I.

TABLE I

CONCENTRATION OF SERUM MAGNESIUM IN NORMAL PERSONS

AUTHORS	PRECIPITATING AGENT	METHOD	RANGE OF VALUE	MEAN
Briggs[3]	Phosphate	Colorimetric	2.23-2.50	
Kramer and Tisdall[4]	Phosphate	Colorimetric	1.80-2.30	2.10
Bogert and Plass[5]	Phosphate	Colorimetric	1.90-2.70	2.30
Watchorn and McCance[6]	Phosphate	Colorimetric	2.30-2.66	2.48
Becher[7]	Phosphate	Colorimetric	1.80-2.30	
Wacker and Fahrig[8]	Phosphate	Colorimetric	2.00-2.97	2.40
Cope[9]	Phosphate	Colorimetric	1.82-2.63	2.06
Walker and Walker[10]	Phosphate	Colorimetric	1.60-3.00	2.20
Brookfield[11]	Phosphate	Colorimetric	1.89-2.19	2.04
Bomskov[12]	Hydroxyquinoline	Bromination	1.70-2.60	
Greenberg and associates[13]	Hydroxyquinoline	Bromination	2.00-3.66	2.74
Velluz and Velluz[14]	Hydroxyquinoline	Bromination	1.60-2.40	2.00
Raices[15]	Hydroxyquinoline	Bromination	1.69-3.00	2.44
Hoffman[16]	Hydroxyquinoline	Colorimetric	1.90-2.50	2.18
Hirschfelder and Haury[17]	Titan yellow	Colorimetric	1.80-2.40	2.11
Haury[18]	Titan yellow	Colorimetric	1.70-3.10	2.33
Bernstein and Simkins[19]	Titan yellow	Colorimetric	1.23-3.54	
Wolf[20]*	Titan yellow	Colorimetric	2.90-4.00	3.61

*For whole blood.

As seen from this table, the mean serum magnesium value of all the authors lies in the neighborhood of 2.4 mg. per 100 c.c. This is in close agreement to the value for serum magnesium of normal persons, as found by Tibbetts and Aub,[50] and corresponds closely to the mean value (2.33 mg.) found in normal medical students, as recently reported by Haury.[18] With the exception of the figures given by Greenberg, Lucia, Mackey, and Tufts,[13] and Bernstein and Simkins,[19] the serum magnesium of normal persons seems to lie between the lower and upper limits of 1.7 and 3.0 mg. per 100 c.c., respectively. Inasmuch as Wolf[20]

determined the magnesium content of whole blood, and since Greenberg and others[13] noted the fact that the red blood corpuscles contain from 5 to 9 mg. magnesium per 100 c.c., we would expect Wolf's figures to be higher than those obtained by other investigators using blood serum or plasma alone. In his review Shohl[21] states that the serum magnesium of normal persons lies somewhere between 1.0 and 3.0 mg. We believe, from our work and from the work of others, that the normal range is from 1.7 to 3.0 mg. per 100 c.c. of blood plasma or serum.

RENAL DISORDERS

Probably the first indication of a disturbance in magnesium metabolism was noted in cases of renal inadequacy. In 1923 Salvesen and Linder[22] reported a series of 15 cases in which the serum magnesium varied from 1.5 to 4.6 mg. per 100 c.c. of serum. In the same year Denis and Hobson[23] reported normal magnesium and potassium values in a series of 19 nephritic patients. They state, however, that the serum calcium was below normal in 5 cases. In view of the inverse relationship which exists between magnesium and calcium, i.e., as the serum magnesium increases the calcium decreases, and vice versa,[11, 24-28] one would be inclined to suspect an elevated serum magnesium in some of the patients studied by Denis and Hobson. In more recent years evidence has accumulated which seems to indicate that retention of magnesium is a common occurrence in cases of renal insufficiency.

In 1932 Becher and Hamann[29] demonstrated that patients with diseased kidneys but with normal renal function had a normal serum magnesium (2.07 mg.). Patients showing a renal insufficiency, however, had a definitely elevated serum magnesium (3.49 mg. per 100 c.c.). In the same year Watchorn and McCance[6] reported 8 cases of uremia in which the serum magnesium ranged from 3.07 to 10.34 mg. per 100 c.c. Two years later Hirschfelder and Haury[30] demonstrated that in nephrectomized animals and in animals with injured kidneys, ingestion of magnesium sulfate caused a rapid increase in plasma magnesium, even to the point of coma. They suggested at that time that "uremic" coma may actually be produced or at least abetted by purgation with magnesium salts. It was pointed out also at that time[31] that a retention of magnesium was a common finding in uremic patients. Numerous papers appeared during the past few years confirming the foregoing findings.[10, 11, 15, 32, 33]

Occasionally a hypomagnesemia may be associated with renal insufficiency. In 1934 Hirschfelder and Haury[31] reported 4 cases of glomerulonephritis in which the serum magnesium was definitely below normal (0.9 to 1.31 mg. per 100 c.c.). In each of these cases either muscular twitchings or convulsions were present. This is of particular interest, since the publication of a report by Kruse, Orent, and McCollum,[1] in which they showed that animals raised on a magnesium-deficient diet have a tendency to become irritable and eventually have severe convulsive seizures. A few years later, Hoobler, Kruse, and McCollum[34] showed that this form of tetany is definitely due to a hypomagnesemia. It now becomes apparent that at least some of the so-called idiopathic tetanies, which cannot be explained on the basis of a low blood calcium, might be accounted for on the basis of a low blood magnesium. Perhaps the case of uremic convulsions described and treated with magnesium sulfate by Watson[35] was actually due to a concomitant hypomagnesemia.

On the grounds of Loeb's[36] pure physiologic work relative to the influence of ions on irritability, we could reasonably conclude that tetany can be due to a deficiency of serum magnesium. From his work we may set up the following relationship: $\frac{Na^{+} + K^{+} + OH^{-}}{Ca^{++} + Mg^{++} + H^{+}}$, as determining the state of irritability. An increase in any of the ions in the numerator, or a reduction of any of the ions in the denominator, will increase the irritability. From this scheme it becomes apparent that a reduction of magnesium is at least one possible factor in the etiology of hyperirritability.

As yet no human pathology has been demonstrated which can be attributed with certainty to a magnesium deficiency. Moreover, since Sherman[37] has shown that the ordinary diet contains more than enough magnesium to meet the daily requirement, it is questionable if a magnesium deficiency ever exists spontaneously in man. On the other hand, in animals we find a disease known as "grass staggers," the symptoms of which are strikingly similar to those seen in experimental magnesium tetany. Sjollema and Seekles,[38, 39] and Metzger,[40] made an extensive study of this form of tetany. They found that the animals become nervous and restless, develop an unsteady gait, and finally die in violent convulsions. Blood chemistry studies in this disease show that the calcium and phosphorus levels are usually low, and that the magnesium is always low. Hopkirk[41] and Cunningham[42] confirmed these findings, and further demonstrated that the symptoms noted in these animals could be overcome by increasing the blood magnesium. It is interesting to note in this connection that Duncan, Huffman, and Robinson[43] produced an experimental tetany in 20 calves fed on whole milk or milk supplemented by iron, copper, manganese, silicon, aluminum, and cod-liver oil. Five of the animals which died in tetany had a normal blood plasma, calcium, and phosphorus, but a low blood plasma magnesium value. The tetanies in the calves due to a low blood magnesium were indistinguishable from those due to a low blood calcium. These could be differentiated only by determining the amounts of calcium and magnesium in the blood. The mean plasma magnesium value of the 5 calves (44 determinations) which died in tetany was 1.6 mg., as compared with the mean value of 2.41 mg. per 100 c.c. in 107 normal calves.[44] McGhee[45] showed that canine hysteria is due to a deficiency in blood magnesium rather than calcium.

We must also consider the possibility that some endocrine organ may control or at least influence magnesium metabolism. This possibility does not seem too hypothetical if one reviews the relationship which exists between sodium and the adrenal glands, iodine and the thyroid gland, and calcium and the parathyroids. Indeed, Coppo and Frugoni[46, 47] believe that the primary action of the parathyroids is to decrease the blood magnesium and that the calcium rises secondarily to antagonize the magnesium. Whether or not hypermagnesemia or hypomagnesemia is due to some endocrine dysfunction must be answered by future investigations.

Other pathologic conditions often associated with a hypomagnesemia will be discussed later under Endocrine Disturbances, Epilepsy, Carcinoma, and Asthma.

DISEASES OF THE CIRCULATORY SYSTEM

Only fragmentary reports have been made on the serum magnesium value in circulatory diseases. In 1923 Weil, Guillaumin, and Weismann-Netter[48] observed an increase in the serum magnesium values of a few cases of essential hypertension. A few years later Blumgarten and Rohdenburg[49] reported 3 patients with arteriosclerosis and one with myocarditis having a high blood magnesium. Unfortunately, their results, obtained on whole blood, are difficult to compare with those of other investigators using serum or plasma. Wacker and Fahrig,[8] Walker and Walker,[10] and Bernstein and Simkins[19] demonstrated only a slight increase in serum magnesium in hypertensive cases not complicated with severe kidney damage. Becher and Hamann[29] showed that the serum magnesium is nearly double the normal value in those patients having hypertension associated with kidney disease. Assuming that all patients with a severe hypertension have also an abnormal kidney function, one would expect at least some elevation in serum magnesium in nearly all cases suffering with this disease.

ENDOCRINE DISTURBANCES

(a) *Diabetes Mellitus.*—Tibbetts and Aub have shown[50] that the ingestion of an acid-producing salt (NH_4Cl) causes a negative calcium balance. Their studies suggest further that magnesium may be used in the body as a substitute for calcium base, the excess usually appearing in the urine. From what we already know of the inverse relationship between calcium and magnesium, we would expect the serum magnesium in such conditions to be definitely elevated. We could speculate then that in cases of diabetic acidosis, as well as in acidosis due to any other cause, a negative calcium balance should exist with a simultaneous elevation in serum magnesium. Our factual information in this direction is entirely inadequate. Brookfield,[11] and Watchorn and McCance,[6] reported three patients with diabetic coma as having a high serum magnesium, whereas a number of similar cases not in coma were said to have a normal serum magnesium. Three of the 7 cases reported by Blumgarten and Rohdenburg[49] were found to have a high and two were found to have a low blood magnesium. None of their patients were in coma.

(b) *Diseases of the Thyroid.*—Blumgarten and Rohdenburg[49] reported 45 patients with goiter on whom blood magnesium determinations were made. In these 15 showed a slight hypomagnesemia and 5 showed a slight hypermagnesemia. Watchorn and McCance,[6] and Becher and Hamann,[29] found normal values in the few cases they reported.

Recently Soffer and co-workers[51a] made an interesting study on the state of the serum magnesium in 31 patients with hyperthyroidism. They found the total serum magnesium in these patients to vary between 1.85 and 2.96, with an average of 2.44 mg. per cent. The total serum magnesium of 14 normal persons varied between 2.12 and 2.76, with an average of 2.52 mg. per cent. However, a striking difference in the ultrafiltrable magnesium was noted. In normal persons this varied between 77.9 and 96.9, with an average of 85.5 per cent. In the patients with hyperthyroidism the ultrafiltrable magnesium was greatly diminished, varying between 38.4 and 78.5, with an average of only 64 per cent. In a

later paper Soffer and associates[51b] made a similar study on an additional 50 patients with hyperthyroidism. Essentially the same results were obtained. In addition they reported that in patients with myxedema, as well as in totally thyroidectomized dogs, the ultrafiltrable serum magnesium is extremely high and frequently is elevated as much as 100 per cent. Since the ultrafiltrable fraction is probably the physiologically important fraction, we could deduce from these results that in hyperthyroidism an actual state of hypomagnesemia exists and that myxedema patients are in a state of hypermagnesemia. This may help to explain the hyperirritability and hypoirritability so commonly associated with Graves' disease and myxedema, respectively. It certainly stresses the importance of determining the amount of physiologically active (diffusible) fraction of this ion before an actual state of hypermagnesemia or hypomagnesemia can be said to exist.

(c) *Diseases of the Parathyroids.*—From the dramatic influence that the parathyroids have on calcium metabolism, we might expect them to have a similar effect on magnesium metabolism. The immediate effect of parathyroid hormone on serum magnesium has been studied in dogs by Scholtz[52] and by Greenburg and Mackey.[53] Their investigations showed that the serum magnesium increased slightly in two to four hours following the injection of the parathyroid hormone. This disappears long before the calcium has reached its maximum value. Bulger and Gausmann[54] noticed that prolonged administration of parathormone to dogs resulted in no striking alterations in serum magnesium. Following the administration of 200 units of parathormone in human beings, Melli and Karadimova[55] observed that the serum magnesium increased during the first hour, reached the maximum at the third hour, and fell to normal at the fourth hour. Serum calcium, on the other hand, began to rise only after the fourth hour. Coppo and Pisa[56] found that one unit of parathormone per day administered to a rat produced an increase in the blood serum calcium and a reduction in the blood serum magnesium. The authors conclude that their analyses furnish an important contribution to the theory that assumes a direct relationship between the function of the parathyroids and the biochemistry of magnesium.[46, 47] Cantarow, Haury, and Whitbeck[57] found that large doses of parathormone injected intramuscularly in dogs produced a slight decrease in serum magnesium in the majority of their experiments. In a human subject Watchorn and McCance[6] also found that intramuscular injections of the hormone produced a slight but definite fall in the serum magnesium.

Denis and Talbot,[58] Bulger and Gausmann,[54] and also Hartog and Muller[32] found a hypomagnesemia in clinical cases of parathyroid tetany. The few serum magnesium studies made in patients with hyperparathyroidism reported by Tibbetts and Aub[59] indicate that a relatively normal serum magnesium prevails in spite of the hypercalcemia. Future investigations in this field should prove most interesting.

(d) *Miscellaneous Endocrine Disturbances.*—Practically nothing is known of the status of serum magnesium in other types of endocrine disturbances. Blumgarten and Rohdenburg[49] found the whole blood magnesium to be within the normal limits in all cases of pituitary dysfunction. Tibbetts and Aub,[61] and

Becher and Hamann,[29] found the serum magnesium to be essentially normal in Addison's disease.

Magnesium may in some way be related to disturbances of the sex glands, especially in the female. In 1926 Watchorn[62] observed variations in human serum magnesium in connection with menstrual irregularities. In 1933 Cannavó and Indovina[63] and in 1935 Dell'Acqua[64] reported that injections of prolan into dogs, cats, and rabbits produced an increase in the serum magnesium. This has recently been denied by Borgstrom.[65]

Zaharescu-Karaman, Alexiu, and Ursu[66] report that the serum magnesium in pregnant women progressively increases as gestation progresses. This is particularly interesting in the light of the findings of Meranze and co-workers[67] that the plasma phosphatase progressively increases during the period of gestation. It is well known since the work of Erdtman[68] that the phosphatase activity of the blood is markedly increased by the addition of magnesium. If the blood magnesium increases during pregnancy, it is understandable why the phosphatase activity should be increased as claimed by Meranze. However, Bogert and Plass,[5] and Frada,[69] reported that the serum magnesium remains within the normal limits during gestation, and Watchorn and McCance,[6] and Wolff,[70] believe it actually falls during pregnancy and remains low during at least the first three months of lactation.

BLOOD MAGNESIUM IN ALLERGIC PERSONS

The relationship of calcium to allergic conditions has claimed a great deal of interest. One group of investigators[71-73] have reported deficiencies of this ion in allergic persons; others[74-78] claim the calcium to be entirely within normal limits.

More recently the closely related companion ion, magnesium, has been implicated in atopy. Choi,[79] Delbet and Palios,[80] Lumiére and Monchal,[81] and Rubin and Rapoport[82] demonstrated that parenteral injections of magnesium salts will protect sensitized animals against the provocative injections of the specific antigen. Lumiére and Monchal,[81] using magnesium thiosulfate, believed that anaphylaxis was inhibited by virtue of the cholesterolemia it produced. Haury[83] observed, however, that if the serum magnesium of sensitized guinea pigs is increased to about 8 mg. per 100 c.c. immediately before the provocative injection of the antigen is made, anaphylactic shock may be prevented. This, Haury believes, indicates that a hypermagnesemia rather than a cholesterolemia is the protective mechanism. The fact that intravenous injections of magnesium sulfate will effectively relieve the bronchial spasms in clinical cases of asthma[84-86] and that magnesium acts to dilate the bronchi when perfused through excised guinea pig lungs[87] may be considered as further evidence that magnesium rather than cholesterol is the antianaphylactic agent.

It has been hypothesized[85] that if a hypermagnesemia will protect against anaphylactic shock, a hypomagnesemia should make one more sensitive to or even incite an anaphylactic reaction. No conclusive evidence is available relative to the serum magnesium values in allergic persons. Fidler[88] in 1934 reported that changes in the blood potassium and magnesium often have a diagnostic value in cases of asthma. Haury[85] has found that 50 per cent of the persons examined during an acute attack of bronchial asthma had a low serum mag-

nesium. Sós and Kunstler[89] claim that allergic diseases (asthma, eczemas, etc.) were found most often in regions where the drinking water was low in dissolved salts. Administration of calcium and magnesium, especially the latter, they reported, seems to be a quick remedy. Braden and Braden,[90] on the other hand, have reported normal magnesium values in 30 allergic persons of various types.

DISEASES OF THE NERVOUS SYSTEM

Epilepsy.—Since the demonstration that convulsions could be produced by a magnesium deficiency,[1, 38-43] it has been considered plausible that a deficiency in blood magnesium might be associated with epileptic convulsions. Although no significant disturbance has been found in the calcium content of the blood and spinal fluid in epileptics,[91-95] there is some evidence accumulating which would indicate that a low serum magnesium may not be an uncommon finding in this malady.

Denis and Talbot[58] reported low plasma magnesium findings in 2 epileptic children, and Blumgarten and Rohdenburg[49] in 1927 found a low serum magnesium in 4 adults with epilepsy. Hirschfelder and Haury[17, 96] noted that the plasma magnesium is frequently low and the potassium is frequently high during the convulsions of epilepsy. McQuarrie and his collaborators[97, 98] also observed an increase in the serum potassium during an epileptic seizure. This latter finding is particularly interesting, since it is known that potassium has an antagonistic action on magnesium.[99] A low blood magnesium content was observed by Wolf[20] in his studies on 100 patients with convulsive states. He also reported that 33 of the 57 patients were helped by small nondehydrating doses of Epsom salts, whereas in the control group taking mineral oil, only 6 per cent showed slight improvement. This is contrary to the findings of Hirschfelder and Haury,[17] who noted the fact that the continuous oral administration of magnesium over a period of three months did not ameliorate the epileptic seizures.

Not all investigators agree that a hypomagnesemia exists during epileptic convulsions. In the 22 cases studied by Greenberg and Aird,[100] the blood serum magnesium remained essentially normal.

Miscellaneous Diseases of the Nervous System.—In 23 miscellaneous diseases of the nervous system, including tabes, chorea, pituitary headaches, and neuroses of various types, Blumgarten and Rohdenburg[49] found the blood magnesium to be essentially normal. Petrunkina[101] reports that the mineral content in the blood of neurotic patients varies more widely than does that of normal persons.

CANCER

Magnesium has been variously implicated in the problem of neoplastic diseases. In 1905 Beebe[102] analyzed human malignant tumors for magnesium and reported that the quantity of it was so small that no quantitative determinations could be made. Clowes and Frisbie[103] reported essentially the same findings in Jensen mouse adenocarcinomas. Robin[104, 105] found insignificant amounts of magnesium in malignant parts of human liver. Later[106] in 3 patients treated with magnesium compounds he observed that the cancerous tissues had the property of fixing magnesium. Eichholtz and Kauderer,[107] in a more recent

investigation, showed that magnesium injected subcutaneously in the form of a salt is stored in the tumor itself. On the other hand, Bolaffi[108] obtained no evidence of such accumulation of this ion in tumors after treatment with magnesium chloride.

Magnesium has been alleged to have an influence on the growth of malignant neoplasms. Sugiura and Benedict[109] believe that the cells of neoplasms, as well as those of normal tissue, require a definite amount of magnesium for their growth. Transplants of the Flexner-Jobling rat carcinoma in rats on a diet containing only 1.8 parts per million of magnesium but otherwise adequate, survived more frequently but grew more slowly than transplants in animals on diets of normal magnesium content. A high magnesium diet, one containing 1,775 parts per million of magnesium, causes a slight increase in the rate of tumor growth and a slight decrease in the number of regressions. Serbescu[110] demonstrated that rabbits which received magnesium sulfate by stomach were much more susceptible to coal tar cancer than were those which did not receive magnesium. In his experiments most of the 30 rabbits which received magnesium orally died within forty days. An equal number of rabbits, not fed magnesium but treated with coal tar in the same manner, lived sixty-three days without developing cancerous lesions. Some of these animals lived longer without being overtaken by cancer. Kreyberg and Nielsen[111] observed that magnesium halides influence the development of tar tumors in white mice. However, they later[112] published another paper in which they claim that the growth of benign and malignant tar tumors in mice is not significantly influenced by the oral administration of mixtures of magnesium salts. That magnesium accelerates the rate of tumor growth (Kyoto transplantable sarcoma) in rabbits is also claimed by Jinguu.[113]

On the other hand, a number of investigators[114, 115] believe that daily doses of magnesium actually retard the appearance or hinder the growth of tar tumors. Bolaffi,[108] McDonald and Itami,[116] Cilotti,[117] Brumpt[118] and others believe that magnesium given either orally or parenterally has no significant effect on the development of tumors.

That a deficiency of this ion may influence the development of cancerous tumors also has its adherents. Klein, Orent, and McCollum[119] claim that a magnesium-deficient diet in rats may produce a possible precancerous condition in the jaw in the region of the molar teeth.

Since 1930 many investigators have made serious attempts to correlate magnesium deficiencies in the soil and water with the incidence of cancer. In Italy, Marchi[120] found that the magnesium content of the salt was in reverse relationship to the death rate from cancer and concluded that cancer was more prevalent in regions where the magnesium content of the diet is low. In France a great deal of significance is attached to magnesium deficiency in the soil as an etiologic factor in cancer. Robinet,[121] and Delbet and Robinet,[122] offered statistics to demonstrate that soils poor in magnesium were accompanied by a high cancer rate. Their statistics, however, were not supported by conclusive analytical data. In Egypt essentially the same idea was advanced by Schrumpf-Pierron,[123] and Bablet and Bader.[124] They concluded that the low incidence of cancer in Egypt was due to the high magnesium content of the soil. Perhaps

if adequate vital statistical records were kept in Egypt, one would find that the incidence of cancer would be very much the same as in other countries.

In 1934 Robinet[125] studied the relationship of the magnesium content of the soil with respect to suicides, tuberculosis, and senility. He concluded that districts with low soil magnesium have a high cancer and suicide rate, and that there are fewer people of old age than in those districts in which the soil is rich in magnesium. In this latter report, as well as in those previously cited, the evidence is far from convincing. Certainly more exact and more extensive soil and food analyses must be made in those localities from which the cancer statistics were taken before any definite relationship can be established between magnesium deficiency and the incidence of cancer.

Shear[126] made a very thorough review of the literature up to the year 1933 on the relationship of magnesium to malignant neoplasms. His conclusions at that time may be quoted to hold true at the present time. "The treatment of cancer patients with magnesium cannot be recommended in spite of the favorable results reported by some clinicians. The analyses of tumors for magnesium are far too scanty to warrant any conclusions"; and finally, "administration of magnesium salts to experimental animals has failed to demonstrate a definite beneficial action and, according to some investigators, has indicated that magnesium may even have a stimulating effect on tumor growth."

MAGNESIUM CONTENT OF THE BLOOD IN CANCER

Far too little data are available at the present time to enable one to draw any conclusions as to the state of the blood magnesium in cancer. Blum and Klotz[127] found the plasma magnesium to be normal in 7 and slightly increased in one cancerous patient. Theis and Benedict,[128] and Blumgarten and Rohdenburg,[49] analyzed the blood of 14 and 17 cases of cancer, respectively. In both instances the magnesium was found to be low in 50 per cent of the cases. Ssawitzki[129] reports that the magnesium of the blood serum (normally 1.6 to 3.5 mg. per cent) is reduced in cancerous conditions by 0.02 to 2.3 mg. per cent. Links[130] believes that variations in the potassium and magnesium content of the serum during the process of its separation from the coagulated blood are significant for the diagnosis of cancer. Knowing the hemoglobin concentration of the blood and the concentrations of potassium and magnesium in three fractions of serum obtained from the same sample of blood during the first hour of coagulation, he believes that he can diagnose the presence or the absence of cancer. In the application of this method in 89 known cases of cancer, he claims to have made 87 correct positive diagnoses. Certainly much more data on blood magnesium and potassium in cancerous patients are needed than we have today before any decisions of such importance can be made. Marx[131] claims that the blood magnesium falls and the calcium rises in experimental osteodystrophy resulting from the administration of parathyroid extract. The fall in magnesium probably is the result of the rise in blood calcium in obeyance of the inverse relationship existing between these two ions, as was previously discussed.

A number of investigators have made claims of a rise of blood magnesium in cancerous patients. Louros and Gaessler[132] obtained a slightly elevated serum magnesium in the blood of 15 women with carcinoma of the uterus.

DISEASES OF LIVER AND GALL BLADDER

The evidence which is available on this subject seems to indicate that a hypermagnesemia is not an uncommon finding in diseases of the liver and gall bladder. This is particularly true if the condition is associated with a frank icterus. In 1927 Blumgarten and Rohdenburg[49] reported the blood magnesium to be elevated in 50 per cent of their patients suffering from liver or gall bladder disease. A few years later Jung[133] found that the calcium content of the serum falls and the magnesium content increases in cases of icterus due to obstruction of the common bile duct. Becher and Hamann[29] noted that patients with icterus due to any cause always have high serum magnesium value.

In this connection it is interesting to note that Rothman, Meranze, and Meranze[134] found the blood phosphatase to be high in cases of obstructive jaundice. The relationship of magnesium to blood phosphatase has been discussed under Miscellaneous Endocrine Disturbances. Lucchi[33] states, however, that a hypomagnesemia is encountered in patients with liver involvement.

MISCELLANEOUS DISEASES

The blood magnesium is said to be elevated in chronic deforming arthritis.[48] Normal magnesium and low calcium values have been reported by Blumgarten and Rohdenburg[49] in this disease. With low calcium values one would expect, however, to find the magnesium elevated.

In typhoid fever, Valdes, Macola, and Segura[135] found that the blood magnesium sometimes is increased slightly while the calcium and phosphorus are usually decreased slightly. Lucchi[33] reports that increases in the magnesium content of the blood were observed in pneumonia and in myelogenous and lymphatic leucemias.

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CLINICAL AND EXPERIMENTAL

THE PHARMACOLOGY OF β (3,5 DI-IODO-4-HYDROXYPHENYL) A PHENYL PROPIONIC ACID*

WALTER MODELL, M.D., NEW YORK, N. Y.

BILISELECTAN† has been used in recent years in Germany for gall bladder visualization.[1-5] Superiority over the radio-opaque phenolphthalein dyes for outlining the gall bladder is claimed because dense shadows may be obtained after oral administration with unpleasant symptoms appearing only rarely. A systematic experimental study of biliselectan has not been published. Inasmuch as the drug may be a useful one the present investigation was undertaken to determine its actions and toxicity.

PHYSICAL AND CHEMICAL PROPERTIES

Biliselectan is β(3,5 diiodo-4-hydroxyphenyl)α phenyl propionic acid. It is a faintly cream-colored powder, insoluble in water, and practically tasteless. It dissolves in alkali, presumably in the form of the sodium salt, when sodium hydroxide is used. Solutions which were used, 2.5 and 5.0 per cent, made up with the minimum amount of sodium hydroxide necessary to bring about complete solution without heat, have a pH of 11.5 or 11.6. These solutions are yellow and clear. A 5.0 per cent solution, kept for forty-eight hours at room temperature in the light, did not show free iodine by the starch test.

EXPERIMENTAL PROCEDURE

Cats were used in several types of experiments, acute and chronic, to study toxicity. The behavior, appearance, and weight of the animals were recorded. Examinations were made for evidence of changes in blood nonprotein nitrogen, red blood cell count, red blood cell fragility, and clotting time. Phenolsulphonphthalein excretion tests were made, and the urine was examined for albumin and reducing substances. These observations were made prior to and for a varying period after the administration of the drug. In the course of the observations on 33 animals, 20 red cell counts, 30 red cell fragility determinations, 20 clotting time determinations, 50 nonprotein nitrogen determinations, 40 phenolsulphonphthalein excretion tests, and 85 urinalyses were made. Most of these tests were made in a series of 12 cats used in chronic experiments.

*From the Department of Pharmacology Cornell University Medical College, New York. Received for publication, February 16, 1942.

†The original proprietary name, Biliselectan, will be used throughout the text, instead of the more cumbersome chemical name. The drug may be secured from the Schering Corporation, which now designate it Priodax.

At autopsy the organs of all animals were examined grossly, and in the case of the animals in the chronic experiments, the livers and kidneys were studied histologically.

All intravenous injections were made with a 2.5 or 5.0 per cent alkaline (pH 11.5 to 11.6) solution, while most of the oral doses were given as the powder in capsules.

TABLE I

EFFECTS OF INTRAVENOUS INJECTIONS OF BILISELECTAN IN UNANESTHETIZED ANIMALS

CAT. NO.	TOTAL DOSE / INDIVIDUAL DOSE*	NUMBER OF INJECTIONS	TIME BETWEEN FIRST AND LAST INJECTION	CONVULSIONS	TIME TO DEATH AFTER FIRST INJECTION	REMARKS
5	150	1	0	Yes	1 minute	
6	285 60/50/75/100	4	4 days	No	Did not die	
7	150 75-75	2	45 minutes	Yes	112 minutes	
8	200 100/100	2	26 hours	Yes	27 hours	Small pulmonary hemorrhages
9	100	1	0	Yes	97 minutes	
10	100	1	0	Yes	46 minutes	
11	500 100-150/250	3	24 hours	Yes	25 hours	
12	250 100-150	2	21 minutes	Yes	Between 12 and 24 hours	Found dead in morning after apparent recovery
13	250 100-150	2	27 minutes	Yes	31 minutes	
14	250 100-150	2	10 minutes	Yes	150 minutes	

*The numbers underneath the total dose indicate the individual doses. Those doses separated by a dash (-) were given on the same day, whereas those doses separated by a line (/) were given on different days.

RESULTS OF EXPERIMENTS

Effect of pH.—Since the intravenous injections of the alkaline solutions of biliselectan were followed by convulsions and other systemic effects, it was necessary to eliminate the alkali itself as a factor in the production of these effects. Therefore, two unanesthetized cats were injected with a solution of sodium hydroxide with a pH (11.5) the same as that of the biliselectan solutions used. Injections of this solution were without noticeable effect even when used in amounts 2.5 times that of biliselectan doses which produced violent convulsions.

Effects of Intravenous Injections on Circulation and Respiration in Anesthetized Animals.—The effect on cardiodynamics and respiration was studied in 4 cats which were anesthetized with sodium amytal and whose blood pressure and respiration were recorded on a kymograph.

Effects were produced which varied from animal to animal. For example, in one animal (Fig. 1) the first injection of 100 mg. per kilogram* produced

*All drugs were administered in relation to body weight, but for the purpose of convenience the expression "per kilogram" will not be repeated.

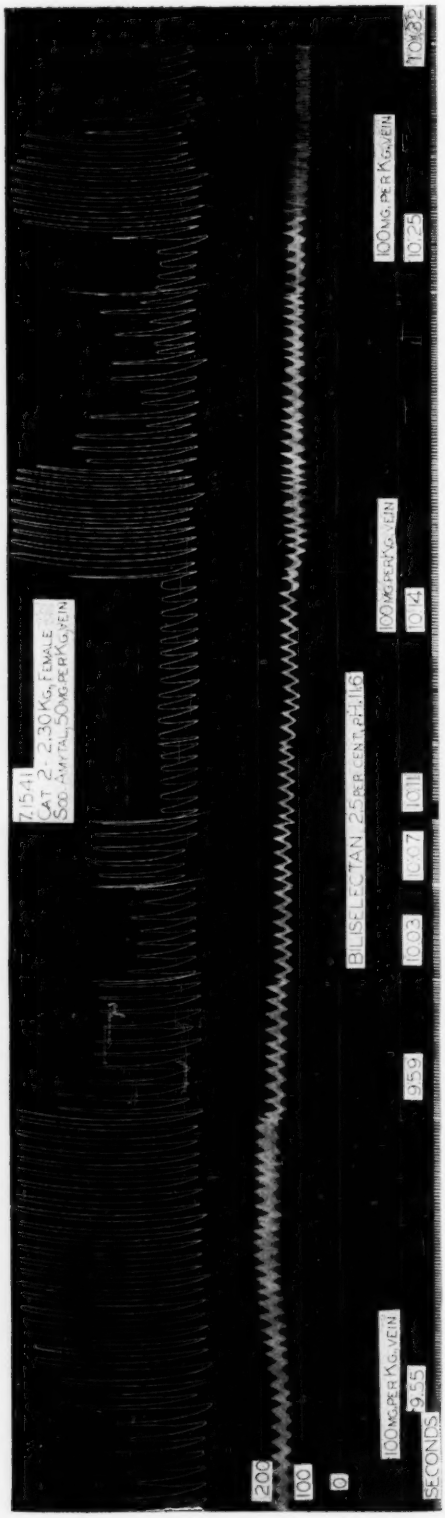

Fig. 1.—Respiratory stimulation produced by biliselectan. Respiration is shown in the upper curve; blood pressure and heart rate in the lower. The calibration lines for the blood pressure indicate millimeters of Hg. The next line in descending order indicates the period of injection, while the last indicates the time in seconds.

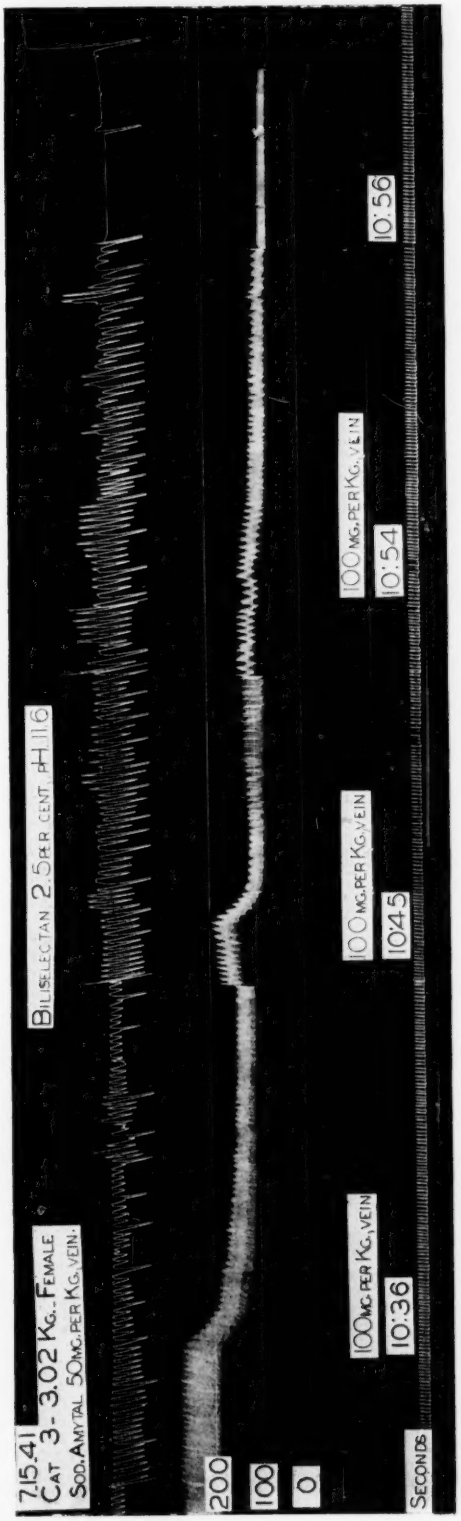

Fig. 2.—Depression of blood pressure, without respiratory depression, produced by doses similar to those shown in Fig. 1.

marked respiratory stimulation lasting almost five minutes. Subsequent similar doses produced progressively less stimulation. On the other hand, in another animal (Fig. 2) receiving the same doses of the same solution there was no respiratory stimulation, but rather a fall in blood pressure after each dose.

Fatal doses were followed by a fall in blood pressure and respiratory depression. In 2 cats the respiratory failure appeared first, the heart continuing to beat after the cessation of breathing. In the other 2 cats the cardiac and respiratory failure appeared simultaneously. In 3 of the 4 cats the terminal events occurred suddenly and without warning.

Effect of Intravenous Injections in Unanesthetized Animals.—In 10 unanesthetized cats the effects of intravenous injections were observed. The significant data are summarized in Table I.

Lethal dose. Single injections of 50, 60, and 75 mg. were not fatal. Injections of 100 mg. killed 2 of 8 cats. A single injection of 150 mg. killed one cat, while another died of the same dose given in two parts within forty-five minutes. Each of 3 cats died of 250 mg. given within thirty minutes in two doses, while one cat survived this dose.

Convulsions. Biliselectan is a convulsant agent similar to picrotoxin or camphor. It produces typical medullary convulsions with all the autonomic phenomena which are associated with it, salivary stimulation, respiratory stimulation and depression when doses of 100 mg. or more are administered. Doses of 50 and 60 mg. failed to induce convulsions, whereas only one of two injections of 75 mg. was followed by convulsions.

The convulsions were always immediate and violent, and without reflex hyperexcitability. Sometimes the convulsions would persist for as long as four to six hours, but neither the duration nor the intensity of the convulsions was an indication of whether the animal would survive a particular dose.

Antidote. The group of four experiments, already described, in which the blood pressure records were made, were performed with amytal anesthesia. In this group the drug proved to be fatal in divided doses of from 200 to 600 mg. without convulsions. The protective action of amytal against biliselectan was studied in another series of 4 cats in which single intravenous doses of from 200 to 350 mg. were administered after 50 mg. of sodium amytal. The two animals which received 200 mg. of biliselectan were protected against the fatal action as well as the convulsions, whereas the cats receiving 300 to 350 mg., respectively, died, although no convulsions were induced. It is to be assumed, therefore, that the fatal effects of biliselectan are only in part the result of convulsions, since with somewhat larger doses, namely, 300 mg., the animals die, even though convulsions are prevented. It is interesting to note that the large doses of biliselectan, through its stimulation of the higher centers, brought about fleeting signs of reversal of the narcotic action of amytal.

Persistence of action. The persistence of biliselectan action is relatively brief. Very little of a given dose appears to remain after twenty-four hours. For example, cat 6 received on successive days injections of 60, 50, 75, and 100 mg. of biliselectan. Each injection was followed by systemic effects: excitement, ataxia, depression, respiratory stimulation, yet the animal recovered

completely after each injection, even the last, although a total of 285 mg. had been administered.

Tissue changes. The post-mortem examination of the organs revealed abnormal findings in only one case. In that there were found minute hemorrhages in the lungs surrounded by small pink areolae. These may have been pin-point pulmonary emboli. Examination for the fragility of the red blood cells was made in 4 cats and no change was found.

TABLE II

EFFECTS OF SINGLE ORAL DOSES OF BILISELECTAN

CAT NO.	DOSE	PERIOD OF OBSERVATION (DAYS)	INTERVAL BEFORE VOMITING	IMPAIRED APPETITE. DURATION (DAYS)	ALBUMINURIA		REMARKS
					DEGREE	DURATION (DAYS)	
15	100	28	No vomiting	None	+	1	
16	250	28	5¼ hours	1	++	1	Less than 10 per cent drug lost in vomiting. Diarrhea for one day
21	250	5	No vomiting	None	+	2	
22	250	2	No vomiting	None	0		
23	250	8	No vomiting	None	0		
17	500	15	20 minutes	1	+	6	About 50 per cent drug lost in vomiting. Pregnant—aborted second day. Depressed for five days
20	500	28	3 hours	1	+	7	Less than 10 per cent drug lost in vomiting. Hematuria and glycosuria for one day
24	500	6	2½ hours	6	++	6	Nonprotein nitrogen rises to 127, cloudy swelling of tubules, marked depression
25	500	2	1 hour*	2	+++	2	
26	500	6	2½ hours*	6	++	4 plus	Died on sixth day. Marked depression
18	1,000	28	7 minutes	2	+	1	About 75 per cent drug lost in vomiting. Diarrhea for one day, depressed for one day
19	1,000	8	No vomiting	8	+	1	Depressed for eight days. Died of pneumonia
31	1,000	1	Vomiting prevented with morphine	1	+++	1	Found dead morning after drug was administered
32	1,000	1		1	?	?	Found dead morning after drug was administered
33	1,000	28		4	++++	5	Glycosuria five days; moderate depression for seven days

*In these animals there was no loss of the drug by vomiting, since there was no expulsion, only retching.

Chronic Experiments—Oral Administration.—The effect of a single oral dose of from 100 to 1,000 mg. was studied in 12 cats for a period of from two to twenty-eight days. The drug was administered usually in capsules after food had been withheld for twelve hours.

The effects are summarized in Table II. There were two deaths which could be ascribed to the biliselectan. Both animals received 500 mg. One died on the sixth day and the other was killed on the sixth day when it was moribund. Another cat which received 1,000 mg. died of a respiratory infection.

Albuminuria was produced in 10 of the 12 cats. It was present in all 7 cats which received 500 mg. or more and in 3 of the 5 cats which received 100 and 250 mg. The albuminuria was transient. It persisted from one to seven days. Glycosuria occurred in one cat, in which it lasted only one day.

Digestive disturbances were common. Vomiting was seen in 7 of the 12 cats; in 1 of 4 which received 250 mg., in all 5 which received 500 mg., and in 1 of 2 which received 1,000 mg. In two instances vomiting was noted seven and twenty minutes after the drug was administered. In the other cases it was delayed for from one to five hours. The appetite was impaired in all the cats which received 500 mg. or more and in one of 5 receiving less. The impairment of the appetite lasted only a day or two in most cats but persisted as long as six to eight days in 3 cats which died. Post-mortem examination did not reveal any evidence of intestinal irritation, but since the intravenous injections did not produce vomiting it was assumed that the digestive disturbances were produced by a local rather than a central action. The loss of biliselectan by vomiting was as indicated in the table, in most cases not sufficient to influence the results.

There was a varying degree of general depression in 5 cats (indisposition to move, lethargy) after doses of 500 mg. or more which usually lasted for from five to eight days.

The average weight of the 4 cats which were permitted to survive for twenty-eight days was 2.42 kg. as compared with 2.61 kg. when the experiment was started. Such a small loss of weight (10 per cent) for cats confined for a four-week experimental period cannot be attributed to the drug.

Biliselectan produced no change in the red cell count, red cell fragility, and clotting time of the blood. In only one cat was there a rise in the nonprotein nitrogen level. None of the phenolsulphonphthalein excretion tests indicated a significant deviation from the control.

The post-mortem examinations and the histologic studies of the livers and kidneys were negative with the exception of one animal discussed below.

The two animals which were killed by the 500 mg. dose of biliselectan suffered from a persistent albuminuria, vomiting, depression, and a loss of appetite which became progressively more profound. One was found dead in a state of decomposition which precluded examination of blood or tissues. In the other the nonprotein nitrogen level in the blood had risen to 127 mg. per cent on the day of death. On the sixth day it appeared moribund and began to vomit again. It was killed in order to obtain satisfactory tissue for histologic study. In the gross examination of the organs there was no significant finding other than the pale kidneys and a small pneumonic patch in the right lung. On microscopic examination of the kidneys, cloudy swelling of the renal tubules was found.

In another series of chronic experiments the complicating factor of vomiting and expulsion of the drug was removed. Three cats were given morphine intravenously to prevent vomiting and a dose of 1,000 mg. of biliselectan orally. Two of the 3 cats were depressed and suffered complete loss of appetite within four hours, and were found dead the following morning. In one of these 2 animals albumin was found in the bladder urine and venous congestion and

parenchymatous degeneration of the tubules of the kidneys on microscopic examination, but no evidence of gastrointestinal irritation. The kidney was normal in the other and its bladder was empty when found so that no urine examination could be performed.

The third cat was slightly depressed for seven days with impairment of appetite for four days. Albumin and glucose were found in the urine for the first five days. There was no significant change in the blood nonprotein nitrogen at any time. After a week the animal appeared entirely normal and continued so for the duration of the experiment (twenty-eight days). On gross and microscopic examination of the organs at the end of this period no pathologic changes were found.

SUMMARY AND CONCLUSIONS

The pharmacology of biliselectan, β (3,5 di-iodo-4-hydroxyphenyl) α phenyl propionic acid, a substance which has been used for gall bladder visualization, was studied in acute and chronic experiments in 33 cats.

By intravenous injection biliselectan acts as a medullary convulsant. Intravenous doses as small as 50 to 75 mg. per kilogram cause excitement with respiratory stimulation. Larger doses cause myoclonic convulsions with stimulation and secondary depression of the circulation and respiration. The intravenous LD 50 ranges about 150 mg. per kilogram. The convulsant action can be abolished by the barbiturate, sodium amytal. In that case, a somewhat larger dose proves fatal by vasomotor and respiratory paralysis without convulsions.

The persistence of action of the intravenous dose appears to be brief, of the order of twenty-four hours or less, as seen by the fact that little cumulation is in evidence when intravenous doses are repeated. However, very large doses by mouth may cause a form of injury which tends to be progressive, so that an animal may die six days after a large oral dose.

The drug is much less toxic by oral administration. By this route animals survive doses as high as 1,000 mg. per kilogram, suggesting that the absorption is very imperfect. Marked individual differences, however, occur. Thus, one animal, after 1,000 mg. per kilogram (vomiting prevented by morphine), showed impaired appetite and slight depression within a few hours after the dose, albuminuria the following day, all of these effects wearing off within seven days; another animal receiving 500 mg. per kilogram showed more severe gastrointestinal symptoms which increased progressively until the animal became moribund and died on the sixth day. Biliselectan given orally does not produce convulsions.

In a total of 15 chronic experiments the effects of single oral doses were observed for periods up to twenty-eight days. Doses of 250 mg. per kilogram or larger cause impaired appetite, nausea, and vomiting and general depression. The intensity of these effects increases with the dose. The emetic effect is due to local action in the gastrointestinal tract, since it does not occur after the intravenous injection. Albuminuria is an outstanding effect. It occurs with doses as low as 100 mg. per kilogram. It is transient, and the duration of this effect varies with the dose.

Doses of from 100 to 1,000 mg. per kilogram are without effect on the nonprotein nitrogen level of the blood, the phenolsulphonphthalein excretion, the red blood cell count, the red blood cell fragility, and the blood clotting time, even though some of the larger doses prove fatal. There are no histologic changes in the kidneys. There were two exceptions, one animal which became depressed after 500 mg. per kilogram, and a nonprotein nitrogen determination during the moribund state (on the day of death) showed a very high level, namely, 127 mg. per cent, and another was found dead the morning after 1,000 mg. In these animals the kidney tubules showed cloudy swelling. Since 1,000 mg. doses failed to cause this effect in 4 other animals, the possibility remains that the blood and renal changes in these cases may have been related to the profound depression of the circulation.

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THE OCCURRENCE OF TRICHINELLA SPIRALIS LARVAE IN TISSUES OTHER THAN SKELETAL MUSCLES*

EVELYN A. MAUSS, SC.D., AND G. F. OTTO, SC.D.
BALTIMORE, MD.

WHILE the larvae of *Trichinella spiralis* develop primarily in the skeletal muscles, there have been reported in the medical literature about 20 fatal cases of trichinosis in which other organs and tissues have been involved. In such cases the myocardium[6] and, to a lesser extent, the central nervous system[4] have been most frequently injured, but larvae have also been reported from the pancreas, kidney, liver, gall bladder, smooth muscles of the intestine, and tissues of the respiratory tract. In addition to the fatal cases there has been reported a far greater number of nonfatal cases with apparent involvement of various organs and systems other than the skeletal muscles. In this connection, we have been particularly impressed by the number of patients who have shown temporary loss of reflexes, and by the number whose spinal fluids have been found positive for larvae.[1, 5]

From our knowledge of the life history of *T. spiralis*, it seems altogether likely that the so-called migrating larvae are carried passively in the blood stream to all parts of the body. Since the larvae have a diameter in cross sec-

*From Department of Helminthology of the School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.
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tion of about 6μ, i.e., less than that of an erythrocyte, it is possible that they are able to negotiate the various capillary beds and to continue uninterruptedly in the blood stream until they reach the skeletal muscles, where they escape from the vascular system, penetrate the muscle fibers, and encyst. A second possibility, however, is that they are carried in the blood to all organs and tissues which they indiscriminately invade, but in none of which, except striated muscle, are they able to develop. Weight is given to this alternative by the cases referred to above, and by reports such as that of Dunlop and Weller[3] in which it was demonstrated that larvae invade the myocardium of rats and are destroyed in situ. The purpose of this study was to ascertain as precisely as possible the extent to which migrating trichina larvae are found in tissues other than skeletal muscles.

MATERIALS AND METHODS

Young mice (5 to 6 weeks old) were infected by esophageal tube with 450 one- to two-month-old trichina larvae freshly isolated from rat muscle by artificial digestion (1.0 per cent pepsin and 0.7 per cent hydrochloric acid). The mice were sacrificed four to fifteen days later and exsanguinated. The blood was collected in sodium citrate, mixed with sufficient distilled water to lyse the cells, and centrifuged, and the sediment was examined for larvae. In attempting to recover the larvae from the tissues of the experimental mice, the foregoing digestion method and the use of other digesting agents failed because the migrating larvae were too young to withstand digestion. Likewise, a modified Baermann apparatus[2] maintained at various temperatures was unsuccessful because the larvae did not migrate out of the macerated tissues and into the funnel. Most of the results reported herein were obtained by direct microscopic examination with 16 mm. objective and 10× oculars, of tissues teased apart and pressed between slides. Attempts at supravital staining in such preparations were abandoned after four commonly used stains (Janus green B, sudan III, Bismarck brown, and methylene blue) failed to facilitate the finding of larvae. The results obtained by direct examination were supplemented by the even more laborious study of serial sections of various tissues.

RESULTS

About 200 slides of serially sectioned tissues of 22 mice were examined. Larvae were seen in the skeletal muscles of animals killed as early as the sixth day after infection. In addition, a few inflammatory foci suggestive of the presence of worms were seen in the myocardium of mice twelve to fourteen days after infection but, since this has been adequately described by Dunlop and Weller,[3] no effort was made to pursue the subject. The direct examination of teased and pressed tissues was on the whole more satisfactory. Unbroken living larvae could be readily seen and identified even in the very young stages, and an entire organ could be examined effectively in much less time than was required for examination of serial sections of equivalent amounts of tissue. Even by this process, however, only 31 larvae were found in addition to those seen in the skeletal muscles and lysed blood (Table I). Of these 31 larvae 24 were recovered from the myocardium. It will be recalled that the methods used did not

permit our determining whether such larvae were free in the tissues or whether they were confined within the vascular system of the organs involved.

In experiments designed to determine whether or not the very young larvae had the capacity of directional movement and could, therefore, respond actively to stimuli, larvae freshly isolated from the uteri of six-day-old female worms were placed on warm, moist slides. Such larvae were observed in more or less continuous motion for several hours, but they never moved from the spot in which they were placed. The addition of freshly teased muscle fibers and of small amounts of other tissues failed to induce directional movement.

TABLE I

OCCURRENCE OF *T. spiralis* IN ORGANS OF MICE INFECTED WITH 450 LARVAE

MOUSE NO.	DAYS AFTER INFECTION	BLOOD (LARVAE/C.C.)	DIAPHRAGM	HEART WALL	LUNG (ONE)	LIVER (ONE LOBE)	KIDNEY (ONE)	TESTIS (ONE)	BRAIN (ONE-HALF)	SPLEEN
501	4	0	0	0	0	0	0	0	0	–
502	5	0	0	0	0	0	0	–	0	–
502a	5	1-2	0	0	0	0	0	0	0	–
503	6	–	+	0	0	0	0	0	0	0
503a	6	10	+	0	0	0	0	0	0	0
504	7	20	++++	2	0	0	0	0	0	0
504a	7	10	++++	1	0	0	1	0	0	0
505	9	+	++++	6	0	2	0	0	0	0
505a	9	+	++++	0	0	0	0	0	0	0
506	10	40	++++	6	0	0	0	0	0	0
506a	10	80	++++	0	0	0	0	0	0	0
507	11	+	++++	4	0	0	1	0	0	0
507a	11	45	++++	0	0	0	0	0	0	0
508	13	50	++++	2	0	2	0	0	0	0
508a	13	100	++++	3	1	0	0	0	0	0
509	14	70	++++	0	0	0	0	–	0	–
510	15	80	++++	0	0	0	0	–	0	–

DISCUSSION

It becomes evident from the foregoing experiments that no great number of *T. spiralis* larvae normally penetrates tissues other than striated muscle in white mice. Despite the afore-mentioned impossibility of determining whether the few larvae that were observed in other tissues had left the vascular system or not, it is interesting to consider the ratio of such larvae to the total number. Since the mice were killed early in the course of their infections, we have no direct measure of the number of larvae which would have developed. In other experiments, however, it was found (Mauss, MSS.) that an average of 26,000 larvae was produced in 13 white mice receiving infective doses of 210 to 1,320 larvae. Using this figure as at least a crude measure of the infections in question, we find that 31 larvae were recovered from organs and tissues other than the striated muscle (or lysed blood) out of a possible final total of 312,000 larvae in the 12 animals killed between the seventh and fifteenth day. This is roughly one in 10,000.

Since it seems unlikely that the situation could be materially different in man than in the white mouse, it would appear that there is usually relatively little trauma in tissues and organs other than skeletal muscles in human trichinosis. Temporary neurologic disturbances or other clinical manifestations may

result from the occasional larvae entering the nervous system or other tissues. In the fatal cases of trichinosis the much heavier infection could increase the likelihood of damage to various organs and tissues without any change in the proportion of larvae invading such tissues. The possibility should not be overlooked, however, that some abnormal distribution of larvae may have operated in those heavier infections of the reported fatal cases.

SUMMARY

Thirty-one migrating larvae were recovered from the carefully examined tissues and organs other than muscles and blood of 12 white mice killed seven to fifteen days after infection with *T. spiralis*. It is estimated that approximately 10,000 times as many larvae are found in the skeletal muscles of mice receiving comparable infections. Apparently, then, invasion of organs and tissues other than skeletal muscles is of minor significance except in peculiarly heavy infections.

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THE TREATMENT OF ACUTE MERCURY POISONING WITH SODIUM FORMALDEHYDE SULFOXYLATE WITH A REVIEW OF TWENTY CASES*

RALPH WOLPAW, M.D., AND NATHAN ALPERS, M.D.
CLEVELAND, OHIO

THERE are two fundamental concepts in the formulation of any method of therapy for acute mercury poisoning. The first is the removal of the unabsorbed mercury as soon as possible in order to prevent further absorption. The second is the removal of the absorbed mercury rapidly with the least toxic effect to the organism.

In cases of oral ingestion the first of these concepts is best carried out by simple gastric lavage, using large quantities of fluid. Thus, any unabsorbed mercury is washed out. If such a procedure has been preceded by vomiting, so much the better, and, when immediate lavage cannot be instituted, fluid should be given after which vomiting should be induced.

*From the Medical Services of the Mount Sinai Hospital, Cleveland. Dr. S. S. Berger, chief of service.

Dr. Wolpaw is now a Captain, Medical Corps, U. S. Army, stationed at Stark General Hospital, Charleston, S. C.; Dr. Alpers is a First Lieutenant, Medical Corps, at Camp Edwards, Mass.

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However, even when vomiting is prompt and lavage is done soon after ingestion, absorption of the mercury is rapid, particularly since it is going through damaged tissue and is causing increased peristalsis by its irritating action. Following absorption, the mercury is distributed relatively equally throughout the body tissues, ordinarily in such small concentrations that it causes no appreciable damage. Excretion begins almost immediately by three major pathways. The first of these is the renal route. The mercury is collected in the kidneys and passes out in the urine. Concentrations of mercury reach a level sufficient to be toxic so that cellular damage (tubular necrosis) results. In like manner, cellular damage (gangrenous colitis) may occur in the colon, which is an important if somewhat later excretory avenue. The third and least important excretory area is the mouth where damage (stomatitis) frequently occurs but is usually not serious. Hence there are three regions in which mercury reaches toxic concentrations and from which it should be removed as rapidly as possible in order to minimize cellular destruction and also to preserve organic integrity.

Of these regions the kidneys are the least accessible and the earliest involved. Direct lavage is technically difficult even in so far as the pelves and calices, where damage is minimal, are concerned, and, at present, technically impossible with regard to the tubules where damage is maximal. Physiologic lavage by massive diuresis is then the method of choice. Fluids in large amounts by mouth and parenterally serve as the best diuretics. Early use of fluids is imperative because the action of the mercury is such as to decrease the excretory powers of the kidneys to the vanishing point (anuria).

The colon is affected by the mercury at first directly by contact and later indirectly by excretion. Early diarrhea is a prominent symptom of acute mercury poisoning and is evidence of the presence of mercury which has passed through from the stomach to the colon. Later, excretion of mercury begins with continuation or renewal of the symptoms of colitis. Here again, the method of choice for treatment is lavage or irrigation in an attempt to wash out the mercury. High colonic irrigations begun immediately and repeated at regular intervals fill this requirement.

Finally, lavage or irrigation of the mouth for both direct and indirect toxic effects of the mercury is indicated in order to remove the metal from this area as rapidly as possible.

The foregoing outline is a very general one of a rational method of treatment of acute mercury poisoning. It is obvious that a neutralizing or antidotal agent for mercury to be used in conjunction with the general method would be of great value. Therefore, after careful consideration of these things, Rosenthal[1] in 1933 reported the results of the use of various antidotes in experimentally produced mercury poisoning in dogs and rats. He advocated the use of sodium formaldehyde sulfoxylate because of its relative stability, low toxicity, and rapid reaction with mercury in vivo and in vitro. He reported the effects of this drug in one human case and outlined a routine of therapy to be followed with the drug. A little later[2] he reported excellent results in 10 human cases following essentially the same routine which he had suggested previously.

This consisted of:

1. Gastric lavage with 5 per cent sodium formaldehyde sulfoxylate, leaving 200 c.c. of solution in the stomach.

2. Ten grams of sodium formaldehyde sulfoxylate in 100 to 200 c.c. of water intravenously in twenty to thirty minutes. If poisoning is severe, this is repeated in four to six hours with 5 to 10 Gm.

3. If colitis develops, high colonic irrigations are to be given once or twice a day with 1:1,000 sodium formaldehyde sulfoxylate.

Brown and Kolmer[3] repeated some of the experimental work on rabbits, and they concluded that sodium formaldehyde sulfoxylate had a definite toxicity which was increased in the presence of mercuric chloride. They felt that there was a certain danger in using the drug intravenously, and the best effects were obtained by oral use as soon as possible. Monte and Hull[4] treated seven cases with the method of Rosenthal, utilizing also physiologic supportive measures. Four of the seven patients showed evidences of severe poisoning, three of them dying. Soon afterward, Rosenthal[5] confirmed the results of Brown and Kolmer, showing that the toxicity of sodium formaldehyde sulfoxylate was increased in the presence of mercuric chloride, but he stated that the increase was not as great as they reported. He suggested that the second intravenous dose should contain 5 Gm. or less of the drug and he showed that the speed of injection affected the toxicity directly. In addition, he cited 25 human cases of his own and about 25 from other clinics in which the results of treatment were good without untoward effects. Following this, in 1937, Modell and associates[6] performed further experiments on animals which had been given fatal doses of mercury. They concluded that the protection afforded by sodium formaldehyde sulfoxylate was limited by the speed of action of the mercury. Moreover, they showed that the end product of the reaction between mercury and sodium formaldehyde sulfoxylate was one-half to one-third as toxic as the mercury itself. Of most importance, however, was their demonstration that the alkalinization of the media increased the speed of reaction of the sodium formaldehyde sulfoxylate with mercury. The last point is of definite significance, especially in cases of oral ingestion of mercury where the poison enters the strongly acid stomach secretion.

Hug[7] reported a series of 72 cases of acute mercury poisoning treated with sodium formaldehyde sulfoxylate by gastric lavage and intravenous injection. There were five deaths, although about one-half the patients showed definite evidence of kidney damage by urinary changes. On the other hand, Monte and Hull[8] reported 40 patients treated with sodium formaldehyde sulfoxylate by lavage and intravenous injection as well as by physiologic supportive measures, such as intravenous glucose and saline solutions, morphine, and transfusions. Eighteen patients developed symptoms of acute poisoning, and of these, 14 died. They concluded that there may be a definite danger in the use of the drug because of the apparent increase in mortality over other averages[9] and also that the drug is valuable only if used very early. Other authors[10-14] have reported cases treated with sodium formaldehyde sulfoxylate by lavage, by intravenous injection, and by colonic irrigation with generally favorable results.

Sodium formaldehyde sulfoxylate is a stable, white, crystalline powder, readily soluble in water. It is relatively nontoxic whether used orally, rectally, or intravenously. It reacts directly with mercuric chloride (more readily in alkaline medium) to precipitate mercury or to form a mercury compound which is one-third to one-half as toxic as the original mercury salt. The last fact accounts for the increase of toxicity of the sodium formaldehyde sulfoxylate in the presence of mercuric chloride. The drug is largely excreted in the urine. Due to these properties sodium formaldehyde sulfoxylate is at present one of the best antidotal agents for acute mercury poisoning. It may be used in all lavage and irrigation fluids as well as intravenously to reach the kidneys. It is easy to handle and it is readily available.

In view of these facts, sodium formaldehyde sulfoxylate was tried in a series of 20 patients over a period of about eighteen months. The routine used was based on that of Rosenthal,[1, 2, 5] with a number of modifications based on other experimental work[6] and on certain personal ideas. It consisted of:

1. Immediate gastric lavage, using 4 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution. Five hundred cubic centimeters or more of solution are used, leaving 250 c.c. in the stomach.

2. Injection of 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously, taking one-half hour for the injection and then following it with a continuous drip of 5 per cent glucose in normal saline. Injection of an additional 100 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously six hours later.

3. Use of two high colonic irrigations a day, using 4 to 5 liters of 1:1,000 (0.1 per cent) sodium formaldehyde sulfoxylate for each irrigation.

4. Gastric lavage with 500 c.c. of 4 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution twice a day for two days.

5. Injection of at least 2,000 c.c. of 5 per cent glucose in normal saline intravenously daily for seven to ten days.

6. In the event of stomatitis, use of a mouth wash of 2 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution two to three times daily.

7. Use of morphine, stimulants, and other supportive measures as needed. If shock is present, it is combatted by appropriate methods.

8. Use of soft diet and forced fluids.

A number of laboratory procedures are performed. Daily routine urine examination is done, and daily nonprotein nitrogen, carbon dioxide combining power, and creatinine determinations are made on the blood. Specimens of urine, stool, vomitus, colonic washings, stomach washings, and mouth washings are examined for mercury daily using the electrolytic method. Naturally, the procedure is modified to fit the needs of the individual patient and in accordance with the clinical course.

All the patients had taken mercuric chloride in doses ranging from 7.5 (one tablet) to 45 (six tablets) grains. The average dose was 17.68 grains and

the mean dose was 15 grains. Emesis occurred in all patients at intervals varying from five minutes to two hours after ingestion. In 13 patients emesis occurred within fifteen minutes. The interval between ingestion and therapy with sodium formaldehyde sulfoxylate ranged between fifteen minutes and thirty-six hours. Sixteen patients were treated within one hour, and 11 within one-half hour (see Table I).

TABLE I

CASE	DOSE IN GRAINS	EMESIS INTERVAL	INTERVAL BEFORE THERAPY	URINE	N.P.N. IN MG. PER 100 C.C.	CREATININE IN MG. PER 100 C.C.	COLITIS	MERCURY PRESENT IN
1	7.5	10 min.	8 hours	Negative	25-30	1.5-1.6	--	Stool
2	15	2 hours	6 hours	1-4+ albumin casts, W.B.C.	25-46	1.6-2.3	Present	Stool, vomitus, urine
3	15	30 min.	45 min.	Trace albumin, few W.B.C.	Not done	Not done	--	Vomitus
4	45	10 min.	30 min.	Trace albumin, W.B.C.	32	1.6	--	Negative
5	15	15 min.	45 min.	1-3+ albumin, R.B.C., and W.B.C.	23-32	1.5-1.6	Present	Stool, vomitus, urine
6	15	10 min.	30 min.	Negative	24-35	1.5	--	Negative
7	22.5	10 min.	20 min.	1-2+ albumin, granular casts	29-44	1.5-1.7	Present	Stool
8	15	5 min.	45 min.	Trace albumin	24-35	1.5	Present	Stool
9	22.5	15 min.	1 hour	1-3+ albumin, R.B.C., and W.B.C.	36-67	1.7-8.3	Present	Stool, vomitus, urine
10	7.5	10 min.	30 min.	Trace albumin	18-26	1.4-1.6	Present	Stool, vomitus, urine
11	30	20 min.	30 min.	3+ albumin, W.B.C., casts	28-93	2.8-13.6	Present	Stool, vomitus, urine
12	45	30-60 min.	3 hours	Not done	Not done	Not done	Present	Not examined
13	7.5	15 min.	15 min.	Negative	21-24	1.5	Present	Vomitus, urine
14	7.5	20 min.	40 min.	Trace albumin	19	1.5	--	Negative
15	7.5	30 min.	30 min.	1+ albumin	28-31	1.5	--	Stool, vomitus, urine
16	7.5	10 min.	36 hours	Negative	28-37	1.5	Present	Stool, urine
17	15	30 min.	30 min.	1+ albumin, W.B.C.	23-34	1.5-1.6	Present	Stool, vomitus, urine
18	7.5	5-10 min.	15 min.	Negative	30-35	1.5-1.6	--	Stool, vomitus, urine
19	30	10 min.	30 min.	3+ albumin, W.B.C., casts	57-121	2.0-7.2	Present	Stool, vomitus, urine
20	15	15 min.	30 min.	Trace albumin, hyaline casts	28-34	1.5	Present	Stool, vomitus, urine

Of the 20 patients 14 (70 per cent) showed evidence of renal damage by changes in the urine, ranging from traces of albumin to 4+ albumin, casts, red and white blood cells. (No urine test was done in one patient who died.) Four cases (20 per cent) showed evidences of renal damage by definite changes in the blood chemistry. No urine examination was done in one patient and no blood chemistry test in two. Thirteen patients (65 per cent) developed colitis, as evidenced by generalized abdominal cramps, and in some cases, bloody diarrhea. Mercury was found in the vomitus, washings, or excreta in 16 of 19 patients examined (84 per cent). In 12 patients (75 per cent of the positive cases) mercury was present in two or more of these groups (see Table I).

Sixteen patients recovered completely with no residual effects. Four patients died. Of these, three developed anuria and uremia, and the other died shortly after ingestion of the mercury in respiratory failure, evidently the result of aspiration of vomited material or edema of the glottis. The case reports of the patients who died are presented here.

CASE 9.—M. W., a 27-year-old white man, swallowed 3 tablets (22.5 grains) of mercuric chloride with a glass of water in an attempted suicide. Fifteen minutes later he vomited blue material, and about one hour later he was brought into the hospital. On admission he appeared acutely ill and he was vomiting bloody mucoid material. His face was blotchy, and his lips, tongue, and pharynx were markedly injected and moderately swollen. Abdomen was tender. He was lavaged with 1,250 c.c. of 4 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution, and he swallowed about 450 c.c. of the same solution. Some of the lavage returns were gray with precipitated mercury. He was then given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously and 100 c.c. of the same solution seven hours later. He was lavaged twice a day for one day, with 4 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate solution, and used a mouthwash and gargle of 2 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate solution two to three times a day. In addition, he was given high colonic irrigations with 1:1,000 sodium formaldehyde sulfoxylate twice a day for three days. In spite of all treatment, including 5 per cent glucose in normal saline intravenously daily, the patient became steadily worse, developed marked oliguria on the third hospital day and slight edema with a moderate temperature elevation on the fourth hospital day. On the morning of the fifth hospital day he developed acute pulmonary edema, even though the intravenous fluid had been changed to 5 per cent glucose in distilled water. He responded well to treatment for this complication, but a short time later he gasped suddenly and died. Permission for autopsy was not obtained. Urines when obtained showed 1 – 3+ albumin and a few red blood cells and white blood cells. Nonprotein nitrogen started at 36 mg. per 100 c.c. and rose to 67 mg. per 100 c.c. Creatinine started at 1.7 mg. per 100 c.c. and at time of death was 8.3 mg. per 100 c.c. Mercury was found in the vomitus, stool, lavage material, and urine.

CASE 11.—H. S., a 28-year-old white woman, took four tablets (30 grains) of mercuric chloride with suicidal intent. She vomited twenty minutes later and was brought into the hospital about one-half hour later. On admission examination revealed an obese woman, appearing acutely ill, vomiting continuously. Her lips were swollen and she showed marked injection of her mouth and throat. Abdomen was slightly tender and showed slight spasm. Remainder of examination was negative. Patient was immediately lavaged with 500 c.c. of 4 per cent sodium formaldehyde sulfoxylate in 4 per cent sodium bicarbonate solution. The washings were dark gray with precipitated mercury. She was washed further with large amounts of normal saline and sodium bicarbonate solution. A continuous intravenous drip of 5 per cent glucose in normal saline was started. Three hours later she was given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously, followed by 100 c.c. six hours later. Because of the character of the washings (containing precipitated mercury), a tube was kept in the stomach for thirty-six hours, and the stomach was washed alternately with plain normal saline and sodium bicarbonate solution and 4 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate solution every three hours. She was given two high colonic irrigations a day with 1:1,000 sodium formaldehyde sulfoxylate and continuous intravenous 5 per cent glucose in normal saline, followed later with 5 per cent glucose in distilled water. She was given mouthwashes of 2 per cent sodium formaldehyde sulfoxylate because of the stomatitis and pharyngitis. On the second hospital day she had an oliguria, and on the third day she became completely anuric. She became progressively worse with onset of drowsiness, edema, and abdominal cramps. She developed hemorrhages in the fundi. Despite diathermy to the kidneys and supportive measures, including sodium lactate intravenously to combat the acidosis, the patient died on the eighth hospital day. Her urine when obtained initially showed 3+ albumin, 4 to 6 white blood cells and occasional casts. Her

nonprotein nitrogen ranged from 28 mg. per 100 c.c. on admission to 93 mg. per 100 c.c. at the time of death; her creatinine from 2.8 mg. per 100 c.c. to 13.6 mg. per 100 c.c. Carbon dioxide combining power decreased to 25.4 volumes per cent, but returned to 41.9 volumes per cent at the time of death. Mercury was found in the vomitus, gastric washings, colonic washings, stools, and urine. Permission for autopsy was obtained. The essential findings at autopsy were:

Gross: Pulmonary edema and patchy bronchopneumonia; large, pale, swollen kidneys, with moderately injected pelves, weighing together 400 Gm.; moderate gastritis, enteritis, and colitis with a greenish gray, pseudodiphtheritic membrane in the rectum and a portion of the sigmoid colon.

Microscopic: Lungs showed pulmonary edema and bronchopneumonia; kidneys showed a marked mercury nephrosis with early regeneration and healing; stomach, small intestine, and colon showed simple inflammation, with slight superficial necrosis of the mucosa in the region of the rectum and sigmoid colon.

CASE 12.—H. B., a 37-year-old white male, took 6 tablets (45 grains) of mercuric chloride with suicidal intent. He vomited thirty to sixty minutes later and was brought into the hospital about three hours after ingestion. Examination on admission revealed a comatose man, breathing heavily and with some difficulty. He was cyanotic and vomited bloody material, and was having bloody diarrhea. Skin was moist and cold. There were coarse râles at both lung bases. Blood pressure was 140/100. He was immediately lavaged with large amounts of 4 per cent sodium formaldehyde sulfoxylate and sodium hyposulfite. He was given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate intravenously, followed by 5 per cent glucose in normal saline in a continuous drip. Preparations were made for transfusion. However, the patient became rapidly and progressively worse and died one hour and forty minutes after admission. Death was evidently due to respiratory failure following either aspiration of vomitus or acute edema of the glottis. It was later discovered that the patient had a history of peptic ulcer. No laboratory studies were done. Autopsy was not performed.

CASE 19.—A. M., a 29-year-old white female, took four tablets (30 grains) of mercuric chloride with suicidal intent. She vomited blue material ten minutes later and was brought into the hospital about thirty minutes after ingestion. On admission examination revealed an acutely ill woman, vomiting large amounts of green fluid and having severe diarrhea. Mouth and pharynx showed considerable injection and swelling. Remainder of the examination was essentially negative. The patient was immediately lavaged with 500 c.c. of 4 per cent sodium formaldehyde sulfoxylate in 2 to 3 per cent sodium bicarbonate solution. About 100 c.c. of solution were left in the stomach. She was then given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate solution intravenously, followed by a continuous drip of 5 per cent glucose in normal saline. Six hours later an additional 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate were given intravenously. A Levine tube was put down into the stomach and the stomach was washed with 4 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate solution every two hours four times. About twenty-four hours after admission the patient was again given 200 c.c. of 5 per cent sodium formaldehyde sulfoxylate solution intravenously and about 500 c.c. of Fischer's solution as well. She was lavaged with 4 per cent sodium formaldehyde sulfoxylate in sodium bicarbonate twice on the day after admission. She was given two high colonic irrigations a day with 1:1,000 sodium formaldehyde sulfoxylate. She was put on a liquid diet with forced fluids and was given large amounts of 5 per cent glucose in normal saline, alternating with 5 per cent glucose in distilled water up to 4,000 c.c. a day. About eighteen hours after admission the patient began to show definite oliguria, and on the fourth hospital day she became anuric. She became progressively worse, appeared toxic and drowsy, and developed a generalized edema on the sixth hospital day and, despite all supportive measures, died on the seventh hospital day. Urines showed 3+ albumin and many granular casts as well as many white blood cells. Nonprotein nitrogen ranged from 57 mg. per 100 c.c. to 121 mg. per 100 c.c., creatinine

from 2.0 mg. per 100 c.c. to 7.2 mg. per 100 c.c.. Vomitus, gastric washings, urine, stools, and colonic washings were positive for mercury. Permission for autopsy was obtained. The essential findings at autopsy were:

Gross: Generalized edema; moderate pulmonary edema and patchy bronchopneumonia; large, pale, swollen kidneys; a deep ulceration in the stomach, about 1 cm. in diameter, with injection of the stomach mucosa elsewhere and moderate injection of the colon.

Microscopic: Lungs showed edema and bronchopneumonia; kidneys showed a marked mercury nephrosis with early regenerative changes; the stomach showed a deep necrotic area, extending into the muscularis, with a mild inflammatory change elsewhere in the stomach mucosa, and the colon showed a mild catarrhal colitis.

COMMENT

The important element in any method of treatment of acute oral mercury poisoning is not the rapidity with which patients recover nor the presence or absence of residual effects, but it is the actual recovery itself or the resulting mortality rate. The mortality rate of 20 per cent obtained in this small series is slightly better than the 25 per cent rate obtained by Hull and Monte[9] in a much larger series treated by various other methods. In the same large series they also noted that about 50 per cent of all cases showed evidence, either urinary or otherwise, of real poisoning, half of these dying. In this series 17 cases, or 85 per cent, showed evidences of poisoning with either renal or colonic damage and 10 cases, or 50 per cent, showed both. Nevertheless, in a small series these figures are not too significant.

In the four fatal cases the doses were larger than the average of 17.68 grains, ranging from 22.5 to 45 grains, the time interval from ingestion to emesis was ten minutes or over, and the treatment interval ranged from thirty minutes to three hours. Moreover, in Case 11 intravenous therapy was delayed for over three hours; hence the absorbed mercury was able to reach the kidneys and cause damage before it could be precipitated. In Case 12 death was primarily respiratory. The importance and value of high colonic irrigations are amply demonstrated by the autopsy findings in Cases 11 and 19 where the colons were only superficially involved, indicating that the irrigations were effective in removing the concentrated mercury. The high incidence of colitis even early in the course of the poisoning makes the prompt institution of high colonic irrigations especially significant. Mercury was found in the stools of 14 of the 16 mercury positive cases, and of the mercury positive cases which showed clinical evidence of colitis it was found in the stools of all except one (11 out of 12). This again emphasizes the role of the colon in the excretion of the mercury.

The importance of early and intensive treatment both oral and intravenous cannot be overestimated, inasmuch as the mercury is absorbed rapidly and produces severe damage in a short time. The fact that the substance formed by combination of mercury and sodium formaldehyde sulfoxylate is still toxic should not be overlooked, so that physiologic supportive measures must be maintained encouraging excretion by all avenues. Moreover, the toxic effect of the mercury on the body as a whole must be combatted by the supportive measures.

The method of treatment advocated here is far from ideal. It is attended by certain dangers and requires careful supervision. Each case should be treated individually and each situation should be met as it arises without too

much dependence being placed on the antidotal drug. However, under careful supervision, the general method has a definite value and the drug deserves further trial until a more specific agent is discovered.

SUMMARY

A method of treatment for acute mercury poisoning utilizing sodium formaldehyde sulfoxylate and based on the work of Rosenthal is outlined. It consists of: 1. Immediate gastric lavage with sodium formaldehyde sulfoxylate. 2. Intravenous injection of sodium formaldehyde sulfoxylate. 3. High colonic irrigations with the same drug twice daily. 4. Gastric lavage twice daily for two days. 5. Daily intravenous glucose in saline. 6. Mouthwash with sodium formaldehyde sulfoxylate for stomatitis. 7. Supportive measures as needed. 8. Fluids and soft diet.

The findings and results in 20 cases treated by this method are presented.

Four fatal cases are presented and the causes of death are analyzed.

The significance of early treatment, physiologic supportive measures, high colonic irrigations, and individual management are stressed.

The method is not considered to be ideal, but it is felt that it should be used in all cases of acute mercury poisoning until a more efficient routine is discovered.

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INFLUENCE OF COMPONENTS OF THE VITAMIN B COMPLEX ON RECOVERY FROM FATIGUE*

E. E. Foltz, M.S., M.D., A. C. Ivy, Ph.D., M.D., and C. J. Barborka, M.S., M.D., D.Sc., Chicago, Ill.

IT IS generally conceded that one of the cardinal symptoms of thiamine deficiency is easy fatigability.[1-4] It is reported that in patients suffering from thiamine deficiency the blood pyruvate is elevated and that the administration of thiamine causes it to become lower.[5, 6] Muscular exercise produces a rise in the blood pyruvate level above that found at rest.[7] Further, thiamine-deficient patients develop a higher level of pyruvic acid in the blood after exercise than do normal persons[8] and are capable of less work.[9] Some investigators[10-12] have felt that the administration of thiamine and other components of the vitamin B complex allayed fatigue. For these reasons, an attempt was made to ascertain whether *recovery* could be hastened by the administration of thiamine and other members of the vitamin B complex to fatigued persons.

Five persons under controlled conditions of housing and diet were studied. The diet was adequate in all respects and contained approximately 1.8 mg. of thiamine and 3.5 mg. of riboflavin. "Double work periods"[13] were used. The work was done on Monday, Wednesday, and Friday afternoons at the same time between 2 and 4 o'clock. The subjects would work to fatigue at a rate of 1,235 kg. per minute, with a pedaling rate of 54 r.p.m. on the bicycle ergometer, described by Kelso and Hellebrandt.[14] The end point was their inability to maintain this rate. Immediately after this first work period the vitamin to be tested was injected intravenously. Then ten minutes after the cessation of the first work period each subject would again work to fatigue. Placebo injections were substituted at random for the vitamin injections. No subject was allowed to watch an injection. The vitamins used were thiamine hydrochloride,† 25 mg., 20 trials; B complex parenteral,† 1 to 5 c.c., 7 trials; cocarboxylase,‡ 10 mg., 4 trials; riboflavin,† 1.25 to 10 mg., 12 trials. The results are shown in Tables I and II.

The results for thiamine, cocarboxylase, and B complex are included in Table I. Since they had no significant effect, the results with each vitamin are

*From the Departments of Medicine and Physiology, Northwestern University Medical School, Chicago.

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Thiamine hydrochloride—50 mg. per cubic centimeter. Riboflavin—1 mg. per cubic centimeter. B complex parenteral, each 10 c.c. contains:

Thiamine hydrochloride, 30 mg.
Nicotinamide, 100 mg.
Riboflavin, 3.3 mg.
Pyridoxine hydrochloride, 10 mg.

‡Supplied by Merck and Company.

not tabulated. The riboflavin was administered at a later period of training, and the data on it are in Table II. Referring to Table I, it will be noted that in 2 of the 3 subjects (B and D) the work output of the second work period following the administration of vitamins is insignificantly less than with the placebo; in the other subject (A) it is not significantly elevated. In no case is the total work output greater following vitamin administration than it is in the control or placebo series. These two factors more than cancel the apparently favorable effect of vitamins on per cent recovery, which effect, however, is not statistically significant.

TABLE I

DATA ON THIAMINE, COCARBOXYLASE, AND THE B COMPLEX

SUBJECT	TYPE OF WORK PERIOD	PERIOD OF TRAINING (MONTHS)	NUMBER OF DOUBLE WORK PERIODS	AVERAGES			
				1ST PERIOD WORK OUTPUT (KG.)	2ND PERIOD WORK OUTPUT (KG.)	TOTAL WORK OUTPUT (KG.)	PER CENT RECOVERY
A	Control	2 -3	19	13,951	6,356	20,275	46
	Vitamin	3 -3.5	9	13,033	7,156	20,168	56
B	Control	1.5 -3.75	21	6,573	4,491	11,142	68
	Vitamin	1.5 -4	15	6,501	4,373	10,889	68
D	Control	2 -3.25	15	10,029	5,595	15,540	56
	Vitamin	3.25-3.75	7	8,816	5,530	14,346	63

TABLE II

DATA ON RIBOFLAVIN

SUBJECT	TYPE OF WORK PERIOD	PERIOD OF TRAINING (MONTHS)	NUMBER OF DOUBLE WORK PERIODS	AVERAGES			
				1ST PERIOD WORK OUTPUT (KG.)	2ND PERIOD WORK OUTPUT (KG.)	TOTAL WORK OUTPUT (KG.)	PER CENT RECOVERY
A	Control	8 -8.5	7	8,788	5,900	14,655	70
	Riboflavin	8.5 -8.75	3	8,355	5,941	14,297	71
B	Control	8.25-8.75	7	6,079	4,260	10,339	71
	Riboflavin	8.75-9	3	6,283	4,384	10,667	70
E	Control	4.5 -5	6	3,770	2,669	6,437	71
	Riboflavin	5 -5.25	3	3,931	2,805	6,736	71
F	Control	4.5 -5	6	4,840	2,696	7,536	56
	Riboflavin	5 -5.25	3	5,254	2,973	8,032	53

Referring to Table II, it is seen that in the case of A the output of work was insignificantly less following riboflavin than in the control series. Considering the subjects B, E, and F, it is apparent that insignificant increases in work output occurred during the first and second periods and in the total work output after riboflavin. However, the insignificant increment observed after the riboflavin can well be explained by training or experimental variation. Also, if an effect had been produced by the riboflavin, a significant increase in the per cent recovery should have occurred. Such was not the case. Thus no significant effects were observed, and the subjects reported no subjective sensations whatever after receiving any of the vitamins.

These results show that the vitamins administered above do not act promptly to facilitate recovery. This statement is made because it is not to be concluded

that an increase in recovery will not occur when these vitamins are added to a diet containing an insufficient quantity of them.

Further Observations on the Double Work Period Method of Studying Fatigue.—One reason for using the double work period method of studying fatigue is that it assists the investigator in determining whether or not his subjects have worked to complete fatigue. This is based in part on our observations[13] that the per cent recovery is more constant than the output of a single work period. For example, if a subject does not work to complete fatigue in the first period, he will work longer than usual in the second period, and his per cent recovery will be correspondingly high. If, however, he does work to complete fatigue the first period and does not work to complete fatigue in the second period, his per cent recovery will be unusually low. To check whether or not per cent recovery could be "faked," four subjects were stopped when they had done approximately 75 per cent as much work as average in the first period. In the second period they were asked to stop as far short of fatigue as they had in the first period. In no case was their usual per cent recovery approximated.

TABLE III

INITIAL DOUBLE WORK PERIODS

(25 Subjects)

	RANGE	COEFFICIENT OF VARIATION (%)
1st period work output	1,112-6,133 kg.	40.9
2nd period work output	761-2,511 kg.	23.0
Total work output	1,873-8,023 kg.	31.1
Per cent recovery	31-99 per cent	19.0

To test the double work period method further, the data of the initial double work periods on 25 new subjects at the above standard rate of work were analyzed. The variability of the four items of fatigue data obtained by this method are shown in Table III. These data indicate that even in untrained persons the per cent recovery is the most constant physiologic characteristic of these four manifestations of fatigue and that the first work period, the variability of which corresponds with that of the single work period, is the most variable. That the ten-minute rest period is not too short to reveal the effect of any substance on recovery has been demonstrated by the favorable effects of caffeine.[13]

CONCLUSIONS

Thiamine, cocarboxylase, riboflavin, and parenteral vitamin B complex given intravenously have no immediate influence on recovery from voluntary muscular fatigue in subjects receiving "adequate" nutrition.

The double work period method of studying fatigue enables the investigator to determine whether his subjects are actually working to a designated end point of fatigue.

The per cent recovery is less variable than the first period work output, the second period work output, or the total work output in initially untrained subjects.

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CLINICAL CHEMISTRY

CIRRHOSIS OF THE LIVER*

Results of Treatment With Parenterally Administered Amino Acids

I. Donald Fagin, M.D., and Francis T. Zinn, M.D.
Detroit, Mich.

PLASMA PROTEINS AND CIRRHOSIS OF THE LIVER

RECENT studies in the relationship between plasma proteins and liver diseases have indicated that in order to maintain an efficient liver function, adequate protein supplies are necessary; and conversely, in order to maintain normal plasma protein levels, the liver must be in a good functional condition.[6]

The finding of decreased concentrations of blood proteins in cirrhosis of the liver was first observed by Grenet and Gilbert in 1907, and has been frequently substantiated since.[1, 2, 8, 10] In general, total protein content of the serum is usually decreased, primarily because of a depletion of the albumin content, and the A./G. ratio is low or inverted. Occasionally, the serum globulin is increased, apparently compensating for the albumin deficiency, so that total serum proteins may be within normal limits.[8, 10] The hypoalbuminemia or hypoproteinemia generally found in hepatic cirrhosis may be attributable to one, or to a combination of the following factors:[11] (a) Deficiency of protein food intake. (b) Protein loss via drainage of albumin in the ascitic fluid. (c) Increased permeability of the capillary bed with consequent diffusion of proteins into the tissues. (d) Failure of the liver to produce proteins or defective formation of proteins. (e) Deficiency of protein-building materials stored in the normal liver. (f) Other factors as yet undefined.

Deficiency of protein intake has been excluded as a significant factor by Foley and co-workers,[10] who described several patients with severe dietary restrictions, but without clinically recognizable hepatic damage, who, although they exhibited some decrease in total proteins, showed no increase in the globulin concentration and no reversal of the A./G. ratio. Myers and Keefer[1] were unable to demonstrate any effect on plasma proteins by feeding patients with cirrhosis of the liver diets containing from 180 to 300 Gm. of proteins daily, possibly because of the impairment of the proteogenic function of the liver.[2, 10] The significance of protein loss via ascitic fluid drainage has been minimized by Barrett, Jones, and Cohn[3] who reported a patient with hepatic cirrhosis who

*From the Medical Service and Laboratories of the United States Marine Hospital, Detroit.
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required frequent paracentesis with removal of an average of 10.2 Gm. of protein per day in the ascitic fluid over a 204-day period, without any definite fall in the plasma protein level. Patients without cirrhosis, but with ascites due to carcinomatous peritoneal or hepatic metastases, or tuberculous peritonitis, or congestive heart failure do not exhibit diminution of plasma protein content, although the protein loss is comparable.[10]

Butt and associates[11] suggested that the usual protein determination does not adequately reflect the osmotic interplay at the capillary membrane. They actually measured the colloidal osmotic pressure of the blood serum in patients with cirrhosis of the liver and found that in almost all cases the serum colloid osmotic pressure was below normal levels. All the patients with edema and ascites had colloid osmotic pressures below the lowest level of normal, probably because of the hypoalbuminemia, since the smaller albumin molecules have the greater osmotic effect. Using the bromsulfalein excretion and hippuric acid synthesis tests to measure the degree of hepatic damage, Butt and his group found an apparent correlation between the colloid osmotic pressure values and the degree of hepatic insufficiency.

The most likely factor, then, to explain hypoproteinemia in hepatic disease is the impairment of hepatic proteogenic mechanism (possibly due to depletion of protein-building stores in the liver), perhaps acting together with increased permeability of the capillary bed.

Pitting edema of the lower extremities is often seen in cirrhosis of the liver, and the most widely accepted explanation has been that the edema is due either to an increased venous pressure in the lower extremities as a result of increased intra-abdominal tension caused by ascites, or to a concomitant congestive heart failure. Myers and Keefer[1] point out that these factors do not explain the dependent edema sometimes seen in patients with a minimal amount of ascitic fluid and without other evidence of circulatory failure. They studied the blood and ascitic fluid protein values in their patients and concluded that the dependent edema is due, partially at least, to hypoproteinemia and increased venous pressure, the latter occurring only in the presence of massive ascites.

RATIONALE

Lillie and associates[7] reported the production of cirrhosis in rats maintained on a diet deficient in protein, and also noted an increase in the severity of the hepatic lesions when 20 per cent alcohol solution was used instead of water in conjunction with the protein-deficient diet. Rats maintained on an adequate protein regimen exhibited no liver changes, even when the 20 per cent alcohol was added. The low sulfur content of the protein-poor diet used suggested a possible relationship between the amino acid content of the diet and the ability of the liver to resist orally administered poisons.

In a later report Lowry and co-workers[12] presented the result of treatment of experimental dietary cirrhosis of rat livers produced by a low choline, low casein diet with added cystine. They found that the use of choline, or a high casein diet, or both, resulted in hyperplastic regeneration of the liver cells, with the disappearance of degenerative fatty changes. Grossly, the livers of rats thus treated exhibited a considerable decrease in size.

It has been shown by several observers (Elman and Weiner, Shohl, Blackfan and Butler; Altshuler, Hensel and Sahyun, quoted in reference 13) that nitrogen equilibrium may be maintained by amino acid mixtures. Messinger[14] has reported serum protein regeneration following the use of amino acids in nephrosis. In view of these observations it was deemed advisable to investigate the effects of administering an amino acid mixture to patients with hepatic cirrhosis to determine what effects, if any, would be demonstrable on serum proteins, edema, ascitic fluid production, liver size, and liver function.

METHOD

Five patients with clinical diagnoses of cirrhosis of the liver were selected at random from the wards; four were in the so-called "decompensated" stage of portal cirrhosis (i.e., they exhibited ascites at some time or another), and one was clinically in the so-called "compensated" stage. The clinical features of these patients are summarized in this report. In all cases jaundice subsided before the institution of the study. In order to obtain a base line, the patients were observed during a period varying from two months to five months, during which time daily weight fluctuations were charted, ascitic fluid output was measured by paracenteses performed as indication arose, blood protein values were determined, and liver function tests were performed. The bromsulfalein excretion and hippuric acid synthesis tests were used.

Other laboratory determinations made included blood nonprotein nitrogen, blood counts, urines (to exclude proteinuria), icteric indices, and sedimentation rates. As an index of impairment of carbohydrate metabolism in hepatic disease, we used the glucose tolerance test (Exton-Rose method)[5] in preference to the levulose and galactose tolerance tests. During the base-line period of observation, the patients were maintained on a diet of carbohydrate 250 Gm., protein 125 Gm., and fat 60 Gm. (The previous diet was the regular hospital diet, which is adequate in all dietary essentials.) After preliminary determinations were completed, the protein was reduced to 80 Gm., being supplemented by 300 c.c. of a 15 per cent solution of amino acids.

Because of the possibility that patients with cirrhosis of the liver might conceivably exhibit disorders of digestion clinically unrecognizable, the amino acids were administered intravenously in order to be certain that they actually reached the liver. The mixture used was prepared from a casein hydrolysate to which tryptophane was added, and conformed to the following analysis:

Total nitrogen	2.0 per cent
Alpha amino acid nitrogen	83 per cent of total nitrogen
Calcium	0.01-0.02 per cent
Tryptophane added	1.0 per cent
Sulfates	0.01 per cent
Phosphates, iron, magnesium	trace
Acidity	pH 4.0-4.3

The solution was administered daily, intravenously, after the patient's breakfasts, for four weeks (except in the case of patient G. R., in whom the development of an incidental type XIII pneumococcus pneumonia with bacteriemia necessitated cessation of the administration of amino acids after three and one-half weeks).

The solution was administered in undiluted form over a period varying from thirty-five minutes to four and one-half hours. Reactions were few and mild. The first dose resulted in an increased number of bowel movements in three of the five patients. The stools were of normal consistency, and the increased number of evacuations was attributed to a hypermotility of the colon. This reaction was observed the first day only and did not recur. Slight nausea, occasional vomiting, or one-half to one degree elevation of temperature, associated with chilly sensations, were occasionally observed during the first week of amino acid administrations. These reactions tended to occur in the patients who were clinically in the poorest condition at the onset of the experiment. The rate of administration of the solution bore no constant relationship to the incidence of such reactions, for a patient occasionally exhibited nausea or a slight fever when the period of administration was four and one-half hours, and no reaction whatsoever when the period was one-half hour. No systemic reactions occurred after the first week of administration of the solution. Local reactions at the sites of intravenous administration of the solution consisted of a slight transient burning sensation if some of the solution leaked into the surrounding tissues, and occasional painless local thrombosis of the veins without associated swelling or inflammatory reaction.

During the month of administration of amino acids frequent determinations of blood proteins were made. The degree of hepatic and splenic enlargement was gauged by measuring the distance of the liver edge or splenic tip from the costal margin in the nipple line. These measurements were made immediately after paracentesis in those patients with ascites to assure greater accuracy.

At the conclusion of the arbitrarily determined month of administration of intravenous amino acids, the bromsulfalein excretion, hippuric acid synthesis, glucose tolerance tests, serum proteins, blood nonprotein nitrogen, icteric index, erythrocyte sedimentation rate, and blood counts were repeated.

CASE REPORTS

CASE 1.—G. R., a 53-year-old white male patrolman, admitted on October 21, 1941, complained of an upper respiratory infection of one month's duration; abdominal swelling, anorexia, exertional dyspnea, and ankle edema of one and one-half weeks' duration. History of left apical tuberculosis, treated from 1929 to 1933 with rest, pneumothorax, and phrenicectomy. The tuberculosis was inactive since 1933. The patient admitted only slight previous indulgence in alcohol. Physical examination: icteric sclerae (icteric index 40); ascites grade 3 (on a basis of severity of grades 1 to 4); grade 1 edema of the legs; diminished breath sounds in both bases; dullness in left apex anteriorly with diminished breath sounds; and liver enlarged to 10 cm. below the costal margin. Grade 4 penile and scrotal edema developed during observation period in hospital. Laboratory data: urine negative; blood Kahn test negative; sputum and ascitic fluid negative for tubercle bacilli; blood sugar and nonprotein nitrogen normal; blood count normal; sedimentation rate 22 mm. in an hour. X-ray examination of the chest revealed thickened pleura of the left lateral chest wall and fibrosis at the left apex with angulation of the trachea. Clinical diagnosis: portal cirrhosis, decompensated; pulmonary tuberculosis, left apex, arrested.

CASE 2.—P. W., a 69-year-old white male seaman, admitted September 17, 1941, had jaundice of ten days' duration, anorexia of one month's duration, and intermittent ankle edema for two years. The patient reported moderately excessive alcoholic indulgence previously. Physical examination: senility; generalized peripheral arteriosclerosis; jaundice (icteric index 75); liver enlarged to 3 cm. below the costal margin; splenic tip barely pal-

pable; grade 3 edema of the lower extremities; slight epigastric tenderness; and an enlarged, tender, boggy prostate. Laboratory data: blood Kahn test negative; urine negative, except for bile during period of jaundice; blood sugar and nonprotein nitrogen normal; blood count normal; sedimentation rate 29 mm. in an hour. Electrocardiogram showed some evidences of myocardial damage. The edema of the lower extremities subsided with rest. Clinical diagnosis: generalized arteriosclerosis and arteriosclerotic heart disease; portal cirrhosis, compensated; chronic prostatitis.

CASE 3.—G. B., a 46-year-old white male laborer, admitted on July 23, 1941, had dyspnea, ascites, and ankle edema of one month's duration. He reported excessive use of alcohol in the past. Physical examination: acutely ill, obese, white male, with scleral icterus (icteric index 25); ascites grade 3; grade 2 edema of the lower extremities; umbilical hernia; bilateral foot drop; liver enlarged to 21 cm. below the costal margin. Laboratory data: urine negative; blood Kahn test negative; blood sugar and nonprotein nitrogen normal; sedimentation rate 21 mm. in an hour. The foot drop cleared rapidly with the use of thiamine chloride, and was attributed to a peripheral neuritis, secondary to previous inadequate dietary intake and chronic alcoholism. During his stay in the hospital the patient developed hematuria (probably due to rupture of a urethral varix) which subsided spontaneously within a few days. Clinical diagnosis: portal cirrhosis, decompensated; chronic alcoholism.

CASE 4.—J. R., a 49-year-old white male laborer, admitted September 22, 1941, complained of ascites of three months' duration, jaundice of three weeks' duration, and ankle edema of five days' duration. He reported exceedingly heavy indulgence in alcohol previously. Physical examination: chronically ill white male; jaundiced (icteric index 145); liver enlarged to 12 cm. below the costal margin; spleen enlarged to 7 cm. below the costal margin; grade 4 ascites; caput medusae; grade 3 edema of the lower extremities and abdominal wall; small umbilical hernia; external hemorrhoids; obesity; and dental caries. Laboratory data: urine negative, except for bile during period of jaundice; blood Kahn test negative; blood count indicated slight anemia; blood nonprotein nitrogen and sugar normal; sedimentation rate 18 mm. in an hour. Clinical diagnosis: portal cirrhosis, decompensated; chronic alcoholism.

CASE 5.—A. G., 52-year-old white male bartender, was admitted October 28, 1941, complaining of anorexia, intermittent nausea, and vomiting, weight loss of 30 pounds, ascites, and weakness, all of six months' duration. He reported moderately excessive previous indulgence in alcohol. Physical examination: generalized icterus (icteric index 18); slight diffuse enlargement of the thyroid; liver enlarged to 11 cm. below the costal margin; spleen enlarged to 7 cm. below the costal margin; minimal pretibial edema; small amount of fluid in the left chest; grade 3 ascites; old gunshot wound of the right lower extremity with an ankylosed right knee; flat feet; varicose veins; and left scrotal hernia. Laboratory data: urine negative; blood count normal; blood Kahn test negative; blood sugar and nonprotein nitrogen within normal limits; sedimentation rate 24 mm. in an hour. The ascites did not recur after initial paracentesis. Clinical diagnosis: portal cirrhosis, early decompensation; left scrotal hernia; old gunshot wound, right lower extremity.

RESULTS

1. *Subjective Effects.*—Clinically, one of the patients (A. G.) reported an excellent improvement in his general condition, associated with moderate gain in strength, great improvement in mental outlook, and an increased feeling of well-being. Three patients (G. R., J. R., and G. B.) reported moderate subjective improvement, and one patient was subjectively unchanged (P. W.). One of the patients (J. R.), however, developed a transitory psychosis three weeks after the cessation of our study, but we believe that this episode represented merely a complicating psychosis in the course of an organic disease rather than any deleterious effect of the therapy per se.

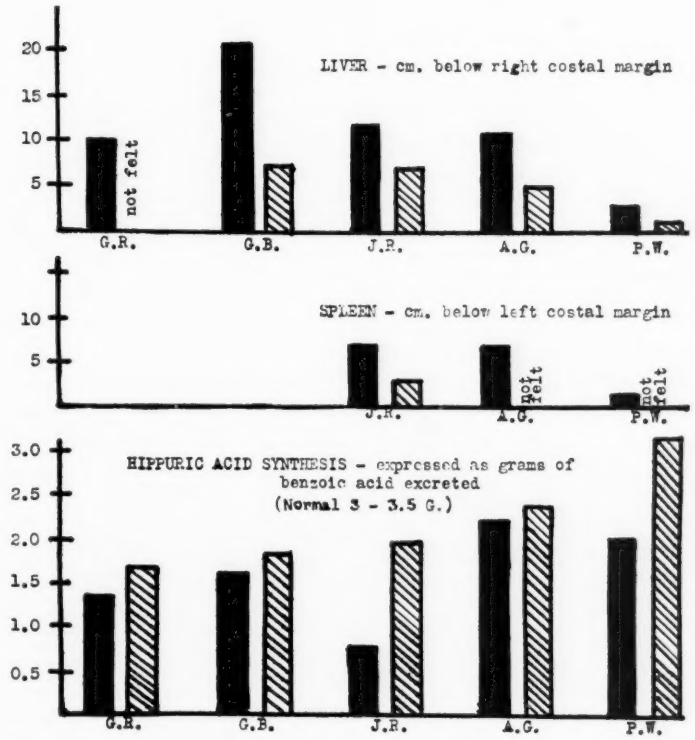

Fig. 1.—The effect of the administration of amino acids on the size of the liver and spleen, and on the hippuric acid synthesis test. Preliminary observations are solid blocks; findings after therapy are represented by lined blocks or the words "not felt."

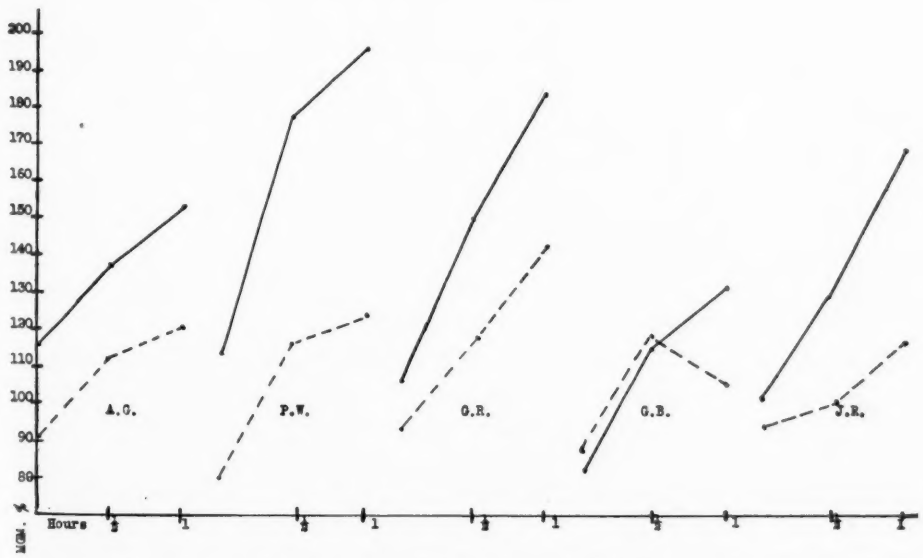

Fig. 2.—The effect of the administration of amino acids on the glucose tolerance curves. Straight lines represent preliminary determinations; broken lines, after therapy.

TABLE I

EFFECT OF AMINO ACIDS ON ASCITIC FLUID

	J. R.	G. B.
Average interval between paracentesis		
Observation period	8.5 days	7.7 days
Administration period	8.7 days	8.7 days
Average daily output of ascitic fluid		
Observation period	1,333 c.c.	1,451 c.c.
Administration period	1,398 c.c.	1,377 c.c.
Average specific gravity of ascitic fluid		
Observation period	1.015	1.011
Administration period	1.011	1.012
Average protein content of ascitic fluid		
Observation period	730 mg. per 100 c.c.	775 mg. per 100 c.c.
Administration period	600 mg. per 100 c.c.	610 mg. per 100 c.c.

2. *Effect on Size of Liver and Spleen.*—The most striking result of the use of the intravenous amino acids was the dramatic decrease in the size of the liver of all the patients, particularly in the four with "decompensated cirrhosis." No decrease in size of the liver or spleen had been noted during the period of observation preliminary to the use of amino acids, and the decrease in size was much more rapid and uniform than that usually encountered in the natural course of the disease. The hepatic and splenic measurements in centimeters below the costal margin before and after treatment are represented in Table II, and in graphic form in Fig. 1.

TABLE II

EFFECT OF THE ADMINISTRATION OF AMINO ACIDS ON THE SIZE OF THE LIVER AND SPLEEN, AND ON THE HIPPURIC ACID SYNTHESIS TEST

	A. G.	P. W.	G. R.	G. B.	J. R.
Liver—cm. below right costal margin					
Preliminary	11	3	10	21	12
After therapy	5	1	0	7	7
Spleen—cm. below left costal margin					
Preliminary	7	1	0	0	7
After therapy	0	0	0	0	3
Hippuric acid synthesis—as grams of benzoic acid excreted (normal 3 to 3.5 Gm.)					
Preliminary	2.20	2.00	1.36	1.60	0.75
After therapy	2.36	3.13	1.66	1.82	1.97

In view of the report of Lowry and associates[12] already mentioned, in which the livers of experimentally cirrhotic rats were observed to shrink in size and exhibit disappearance of degenerative fatty changes when treatment with choline or a high-casein diet was instituted, it seems logical to assume that the diminution in size of the livers in these cases is primarily due to resorption of the fat in the liver cells. The decrease in the size of the spleen in those patients who exhibited splenomegaly may be attributable to circulatory readjustments secondary to the decreased size of the liver. The mechanism underlying the resorption of the fat in the liver cells is uncertain, but it probably represents a lipotropic action of the

administered amino acids. Unfortunately, we were unable to follow up our patients with liver biopsies.

The possibility that the shrinkage in the size of the liver reflects a change in the water content of the liver tissue is worthy of consideration, and animal experimentation is necessary to confirm or refute this aspect.

3. *Effect on Peripheral Edema.*—Four patients presented peripheral edema of varying degree at the initiation of the study, and all evidenced complete resorption of the edema fluid within two weeks after the start of amino acid administration. We are inclined to believe that this change reflects improvement in the serum proteins and osmotic balance at the capillary membranes.

4. *Effect on the Ascitic Fluid Output.*—The administration of amino acids had no appreciable effect on the output of ascitic fluid, or on the specific gravity of the ascitic fluid. Only two patients required paracentesis a sufficient number of times to warrant consideration, and the characteristics of the ascitic fluid in these patients is presented in Table I. It will be noted that there is a slight but appreciable decrease in the average protein content of the ascitic fluid after the administration of amino acids. We do not believe that the changes in the amount of fluid, or in the interval between paracentesis, or in the specific gravity of the ascitic fluid before and after therapy are of significance. However, the decrease in average protein content of the ascitic fluid during and after therapy may reflect improvement in serum proteins and capillary permeability.

5. *Laboratory Findings.*—(a) Effect on serum proteins: Serum proteins were determined by the use of photoelectric colorimeter, as described by Looney and Walsh.[9] In our hands the results were too bizarre and variable to warrant consideration. Despite the fact that a large part of the study depended upon our following the serum proteins, we were unable to draw any conclusion thereunto appertaining because of the inaccuracy of the method in our hands. Nevertheless, other findings of sufficient importance developed during the course of the study to warrant presentation of our results.

(b) Effect on hippuric acid synthesis: Reference to Table II indicates that in all cases there was some improvement in the ability of the liver to handle ingested sodium benzoate. In three cases (G. R., J. R., and P. W.) this improvement is of significant degree. These findings are suggestive of a beneficial effect on the detoxification function of the liver, and possibly an improvement in the ability of the liver to manufacture the amino acid glycine, since the hippuric acid test indicates the ability of the liver to conjugate ingested sodium benzoate with glycine, forming hippuric acid, which is excreted in the urine if renal function is adequate.[4]

(c) Effect on glucose tolerance curve: Reference to Fig. 2 indicates that in every instance the glucose tolerance curve changed somewhat in the direction of improved utilization of glucose after the period of amino acid administration. We realize that these tests are subject to criticism in that they represent single examinations and not a summary of daily variations in the glucose tolerance of the individual patient. However, the fact that they all changed in the same direction leads us to believe that intravenous administration of amino acids pro-

moted increased tolerance and utilization of carbohydrates, particularly since the patients were on an adequate diet when the initial determinations were made.

(d) Effect on bromsulfalein excretion: In only one patient (G. R.) was there any appreciable effect on the bromsulfalein excretion. In this patient, prior to the administration of amino acids, there was 40 per cent bromsulfalein retention in the blood stream after one hour, and at the conclusion of the period of intravenous amino acid administration there was only a 12 per cent one-hour retention. The other patients exhibited no significant changes in bromsulfalein excretion.

(e) Effect on other blood constituents: The blood count, nonprotein nitrogen, sedimentation rate, and icteric indices exhibited no significant changes during the course, or at the conclusion of our period of study. As has been noted, clinical jaundice had subsided in all these patients before the study was begun.

SUMMARY

1. A study of the effects of the intravenous administration of amino acids to five patients with cirrhosis of the liver is presented.

2. Clinically, one patient exhibited great general improvement; three exhibited moderate general improvement, and one remained unchanged.

3. The most striking result was shrinkage in the size of the liver and spleen, possibly attributable to resorption of intracellular fat in the liver, with circulatory readjustments secondary to the improved portal circulation resulting in decrease in splenic congestion.

4. Peripheral edema subsided in all patients exhibiting it within two weeks after the initiation of amino acid therapy.

5. Laboratory data accumulated during a preliminary period of observation and at the conclusion of a period of amino acid administration indicated an improvement in glucose tolerance and in hippuric acid synthesis in all patients, and an improvement in bromsulfalein excretion in one patient.

6. Further study and corroboration is needed to determine the degree of benefit obtainable by the use of amino acids in cirrhosis of the liver.

We wish to thank Mr. Raymond L. Charles, chief technician, for his valuable assistance.

The amino acids solution used for this investigation was Amino Acids Parenteral Stearns, supplied through the courtesy of Frederick Stearns & Company, Detroit, Mich.

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LABORATORY METHODS

GENERAL

A STUDY OF THE PHENOMENON OF ERYTHROCYTE SEDIMENTATION*

II. The Establishment of a Reliable Measurement of the Phenomenon—Its Reproducibility and Limitations

Roy E. Nichols, B.V.Sc., M.Sc., Ph.D.,† Lafayette, Ind.

IN A PREVIOUS publication[1] literary evidence of the source of my conception of the status of sedimentation was advanced. It early became evident that choice or development of a suitable technique for the measurement of sedimentation would be a difficult task indeed since there were and still are in use a multiplicity of techniques, many quite different.

The horse was chosen as the experimental subject since large samples of blood could be procured with ease and at fairly frequent intervals. Agglutination of equine red cells and thus sedimentation are very rapid even in healthy subjects. Probably because of its natural rapidity, separation of equine corpuscles seems somewhat more sensitive to such physiologic factors as fear and hard work and such technical factors as delay, degree of agitation, and anticoagulation. It is felt that this greater sensitivity is a desirable quality because factors which are responsible for variations in sedimentation are more easily discerned and their control is more clearly demonstrated.

Many hundreds of tests were conducted comparing many different techniques and modifications of techniques until finally one was observed that would yield quite comparable results upon repetition, and, since measurement was continuous, was thought to be reasonably complete. With the exception of a description of the technique finally obtained and the suggestion of a more practical "field" technique, together with evidence of the reproducibility and limitations of each, inclusion of the various results of this labor hardly seems necessary here.

EQUIPMENT

For collecting bottles ordinary ½ ounce homeopathic vials are measured and scratched at 5 c.c. and 10 c.c. levels. Corks are paraffined. The desired quantity of anticoagulant is added to each vial by first preparing a weak solution of the chosen anticoagulant, adding the volume necessary to each vial and allowing the water to evaporate in the incubator. For routine work a solution of 1.2 per cent ammonium oxalate and 0.8 per cent potassium oxalate is prepared in dis-

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†Comparative Physiologist, Department of Veterinary Science, Purdue University.

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tilled water. One cubic centimeter of this solution when evaporated leaves 12 mg. of ammonium oxalate and 8 mg. of potassium oxalate and is suitable for 10 c.c. of blood—0.5 c.c. of this solution for 5 c.c. of blood. An ample reserve of previously prepared vials is kept on hand.

A sharp clean dry 15 gauge 2 inch hypodermic needle and a clean dry glass syringe of the capacity most suitable are usually employed.

Pledgets and some suitable disinfectant for preparing the area for venipuncture are necessary.

An incubator regulated at body temperature is utilized.

Wintrobe modified hematocrit tubes[2] are employed routinely.

For filling the tubes routinely 18 gauge 4½ inch spinal puncture needles, with round hubs and with the tip ground flat, are used. All filling needles are fitted with rubber medicine dropper bulbs of fairly uniform consistency and capacity. Sixteen gauge 6 inch needles have been more recently used.

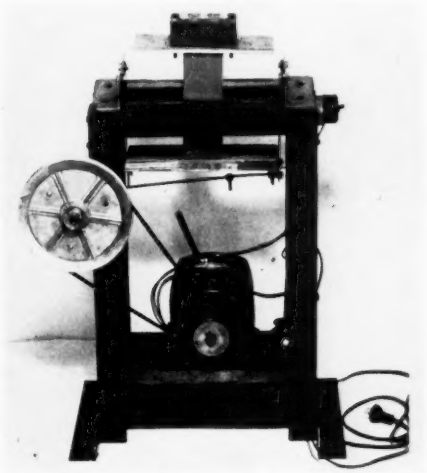


Fig. 1.—Shaking apparatus.

For shaking the blood sample previous to filling the Wintrobe tube a mechanical agitator driven by a variable speed motor has been made. The rack for holding the collecting vials is suspended from four 7¾ inch spring steel arms. This rack is driven over a distance of ¾ inch by a spring steel arm fastened to the bottom of the rack and to a balanced eccentric at the end of a jackshaft. At present the speed of the motor is adjusted to move the rack approximately 564 strokes per minute (Fig. 1).

An automatic recording device employing continuous photography (reported in a previous publication[3]) was made, utilizing the fact that cells cut out most of the light coming through the Wintrobe tube while plasma does not. An image of a very narrow vertical portion of a blood-filled tube is transferred by a photographic lens onto sensitized paper attached to a revolving drum. As the cells settle out more of the sensitized paper is exposed. Results with this apparatus are illustrated in Fig. 3.

Eastman P.M.C. No. 1 normal bromide paper of single weight is used routinely. For developing and fixing solutions those used for ordinary x-ray work are very satisfactory.

A millimeter rule and a simple cardboard "trigonograph" (Chemical Rubber Company) are of great assistance.

The centrifuge employed is an International No. 1 type SB with a head that accommodates eight 15 c.c. conical centrifuge tube holders. The distance from the center of the shaft of the centrifuge to the center of each trunnion ring is 3½ inches. The speed when the rheostat arm is set at mark "17" is approximately 3,000 r.p.m. with 6 filled Wintrobe tubes. Rubber cushions cut from corks are made for the bottom of the centrifuge tube holders. Side cushions are made from 3 inch lengths of ⅛ inch wall, ⅜ inch bore rubber tubing.

A rack for placing tubes in a vertical position for the "field" technique was made by machining parallel V-shaped grooves on the side of a brass casting. These grooves can be rendered perpendicular by adjustable legs, using as a guide a special plumb bob fastened to one end of the machined casting. The sides of the tubes are held against the sides of the V-groove by spring brass clips (Fig. 2).

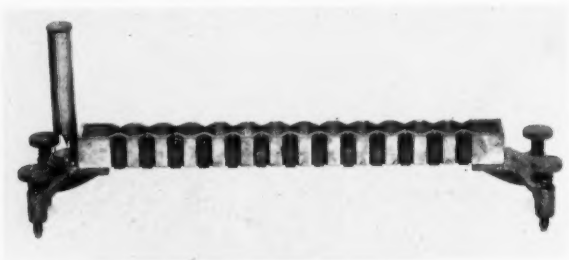


Fig. 2.—Perpendicular tube rack.

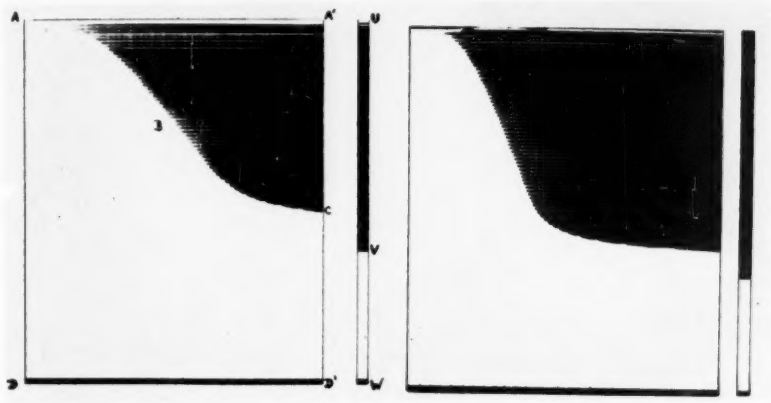
DESCRIPTION OF TECHNIQUE

When all minor details pertaining to equipment, restraint, disinfection of the area, etc., have been taken into consideration, the jugular vein of the animal chosen is raised by thumb pressure. As soon as the needle is in the vein, this pressure is completely released and, after a second's hesitation to allow the establishment of free flow in the vein, the desired quantity of blood is withdrawn into a syringe. After obtaining this desired quantity of blood in the syringe, the collecting vial (or vials as the case may be) is immediately filled to the necessary mark, corked, and is thoroughly agitated immediately to mix the blood and anticoagulant. Of course, the syringe must be washed before clotting can take place.

The collecting vial (or vials) is then placed in a quiet place until the cells have settled, which with horse blood usually requires anywhere from five to twenty or twenty-five minutes. Next the vial (or vials) is placed in the mechanical shaker for fifteen minutes. While the shaker is still in motion, the cork is taken out, a quantity of blood is removed from the bottom of the vial by the filling needle and bulb, and a Wintrobe tube is carefully filled to the "100 mm." mark *avoiding forceful filling* and bubbles. The tube is then placed in the tube holder of the automatic recorder and the shutter is opened. Recording is allowed to take place for whatever time the sample indicates—usually thirty minutes from the time of filling the tube. If settling is not fairly complete, a longer time

can be chosen but routinely this is rarely necessary. After recording, the same sample can be centrifuged (about 3,000 r.p.m. per thirty minutes), and the results are either photographed on the same record or read off directly from the tube.

After the picture is exposed, developed, and dry, measurements of whatever sort desired can be made.



Ex. 1. Ex. 2.

Fig. 3.—Examples of results as obtained with the photographic sedimentation recorder.

Examples 1 and 2 are results obtained by the photographic recorder, example 1 being labeled for explanatory purposes (Fig. 3):

DD′ is the top of the dark heavy line and represents the bottom of the column of blood. The length of DD′ (or AA′) represents the time of exposure.

AD represents the height of the column of blood (100 mm.) at the start of the measurement when the cells are dispersed throughout the plasma.

The dark area ABCA′ represents the exposed area of the graph brought about by the increasing amount of light passing through the plasma as the cells settle out.

The light area ABCD′D represents the unexposed area brought about by the presence of cells through which the light cannot pass.

AB represents the beginning and gradually accelerated descent of the line of demarcation between cells and plasma.

BC represents the gradually retarded descent of the line of demarcation between the cells and plasma brought about by the gradually increasing effect of heaping of cells in the lower portion of the tube.

B represents the theoretical point of change between the gradually accelerated descent (AB) and the gradually retarded descent (BC) of the line of demarcation between cells and plasma.

Sigmoid curve ABC then represents a graphic record of the descent of the line of demarcation between cells and plasma for a given time.

The small light horizontal lines in area ABCA′ represent the millimeter divisions on the tube.

The small vertical lines in area ABCA′ occur at intervals of approximately 1.02 minutes as the apparatus is now run.

The narrow dark vertical lines preceding and following exposure of the sedimenting and centrifuged sample are made by slight exposure of the sensitized paper when the tube holder is empty. These are essentially not necessary but are handy.

UW represents the height of the column of blood (100 mm.).

UV represents the height of the column of plasma after centrifugation (volume percentage of plasma).

VW represents the height of the column of cells after centrifugation (volume percentage of cells).

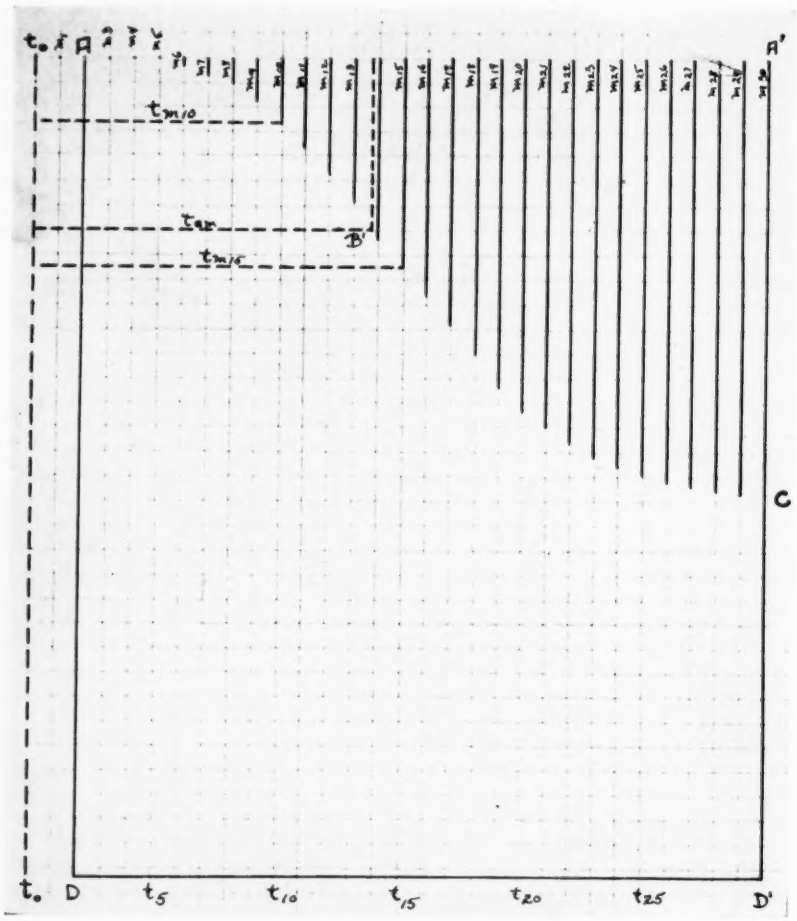


Fig. 4.—Ex. 1 of Fig. 3 plotted graphically.

To clarify some of the measurements made from the afore-mentioned photographs the following graph of example 1 is presented in Fig. 4:

1. Line t_0–t_0 is located by subtracting the time of filling the tube from the time that the exposure is completed, multiplying by 3 (since 3 mm. are the equivalent of one minute on the photograph), and then laying this distance off from A′D′, the end of the exposure. Line t_0–t_0 then represents the time of filling of the tube. In some of these examples t_0–t_0 is purposely quite a distance

from AD, this to separate t_0–t_0 from AD to make a clearer graph. In practice t_0–t_0 is usually not more than fifteen to thirty seconds from AD.

2. Times (t) are measured in minutes from t_0–t_0 as t_1, t_5, t_7, etc., subnumbers representing the number of minutes from t_0–t_0 (3 mm. of horizontal distance equal one minute).

3. Perpendicular distances (m) from line AA′ to curve ABC are measured in millimeters as m_3, m_5, and m_7, etc., the subnumbers representing times in minutes from t_0–t_0 that these distances are measured. m is affected by variations in filling the tube to the "100 mm." mark and by variations in image size. However, these are usually very small and can be allowed for during measurement.

4. B′ is the estimated point of change between the vertical flexion (AB′) of curve AB′C and the horizontal flexion (B′C). With care and experience this point can be very accurately adjudged, especially if the curve is tilted in such a way that the eye can follow an imaginary tangent to the steepest portion of curve AB′C. Assistance in judging this point is also furnished by applying a straightedge as a tangent.

5. em is the perpendicular distance from AA′ to B′. This measurement is subject to the same variations as m. In Ex. 1: 21 mm.

6. t_{em} is the horizontal distance from t_0–t_0 to B′ divided by 3 (since a horizontal distance of 3 mm. on the photograph represents one minute) and expressed as minutes. Ex.: 13.8 minutes.

7. $\angle_g$ is the least angle between the apparently steepest slope of curve AB′C and m. This angle can be measured by lining up AD with one side of a trigonograph and moving the free arm of the trigonograph until it lies tangent to the apparently steepest slope of curve AB′C, this being very closely related to the A side of B′. Considerable error can be observed if the free arm of the trigonograph is lined up with too great a distance on curve AB′. Careful and close scanning will allow use of as little as 1 to 2 mm. on curve AB′ as a tangent to the free arm. Ex.: 32° 20′.

8. I_{em} is an index calculated by the formula: $\frac{em}{t_{em} \times 3}$.

$$\text{Ex.: } \frac{21}{41.5} \text{ or } 0.51.$$

If one-minute readings of the height of the column of cells are made, as for instance in Ex. 1:

t_0 = 100 mm.	t_8 = 97 mm.	t_{16} = 71 mm.
t_1 = 100 mm.	t_9 = 94½ mm.	t_{17} = 67½ mm.
t_2 = 100 mm.	t_{10} = 92 mm.	t_{18} = 64 mm.
t_3 = 100 mm.	t_{11} = 89 mm.	t_{19} = 60 mm.
t_4 = 100 mm.	t_{12} = 86 mm.	t_{20} = 57 mm.
t_5 = 100 mm.	t_{13} = 82½ mm.	t_{21} = 55 mm.
t_6 = 99 mm.	t_{14} = 78 mm.	t_{22} = 53 mm.
t_7 = 98 mm.	t_{15} = 74½ mm.	t_{23} etc.

and if these are subtracted from the height of the column of blood (usually 100 mm.), values of m will be:

t_0 = 0 mm.	t_8 = 3 mm.	t_{16} = 29 mm.
t_1 = 0 mm.	t_9 = 5.5 mm.	t_{17} = 32.5 mm.
t_2 = 0 mm.	t_{10} = 8 mm.	t_{18} = 36 mm.
t_3 = 0 mm.	t_{11} = 11 mm.	t_{19} = 40 mm.
t_4 = 0 mm.	t_{12} = 14 mm.	t_{20} = 43 mm.
t_5 = 0 mm.	t_{13} = 17.5 mm.	t_{21} = 45 mm.
t_6 = 1 mm.	t_{14} = 22 mm.	t_{22} = 47 mm.
t_7 = 2 mm.	t_{15} = 25.5 mm.	t_{23} etc.

To continue the definitions:

9. M_1 is the maximum difference in millimeters between any two consecutive minute readings of m. In the above example, 4½ mm.

10. t_{M_1} symbolizes the times in minutes from t_0–t_0 between which M_1 occurs. In the above example, thirteen to fourteen minutes.

11. I_{M_1} represents an index range calculated by dividing the values of m between which M_1 occurs by their corresponding times multiplied by 3, since 3 mm. is the equivalent of one minute. Ex.: $\frac{17.5}{39} - \frac{22}{42}$ or 0.45 – 0.52.

12. $\angle_{M_1}$ is measured by calculating on the trigonograph the angle opposite the horizontal side of a right triangle having M_1 as the perpendicular side and 3 mm. (one minute) as the horizontal side. Ex.: 33° 55′.

13. M_5 is the maximum difference in millimeters between any two consecutive five-minute readings of m starting at t_0–t_0; i.e., the maximum five-minute fall of the line of demarcation between cells and plasma. Ex.: 17.5 mm.

14. t_{M_5} symbolizes the times in minutes from t_0–t_0 between which M_5 occurs. Ex.: ten to fifteen minutes, fifteen to twenty minutes.

15. I_{M_5} represents an index range calculated by dividing the values of m between which M_5 occurs by their corresponding times multiplied by 3, since 3 mm. are the equivalent of one minute. Ex.: 0.27 to 0.47, 0.57 to 0.72.

16. $\angle_{M_5}$ is equivalent to the angle opposite the horizontal side of a right triangle having M_5 as the perpendicular side and 15 mm. (five minutes) as the horizontal side. Ex.: 40° 40′.

If readings are made of m every five minutes, starting at times other than t_0–t_0, we find differences in M_5, I_{M_5}, and $\angle_{M_5}$:

Time	M_5	t_{M_5}	I_{M_5}	$\angle^s_{M_5}$
t_{0-5}	17½ mm.	15-20 min. 20-25 min.	0.18-0.43 0.43-0.57	40° 40′
t_{0-4}	18 mm.	15-20 min.	0.24-0.48	40°
t_{0-3}	18½ mm.	15-20 min.	0.31-0.54	39° 10′
t_{0-2}	18½ mm.	15-20 min.	0.39-0.5	39° 10′
t_{0-1}	18 mm.	15-20 min.	0.49-0.67	40°
t_0	17½ mm.	10-15 min. 15-20 min.	0.27-0.57 0.57-0.72	40° 40′
t_{0+1}	18 mm.	10-15 min.	0.37-0.64	40°
t_{0+2}	18½ mm.	10-15 min.	0.47-0.72	39° 10′
t_{0+3}	18½ mm.	10-15 min.	0.58-0.80	39° 10′
t_{0+4}	18 mm.	10-15 min.	0.73-0.89	40°
t_{0+5}	17½ mm.	5-10 min. 10-15 min.	0.53-0.85 0.85-0.96	40° 40′

To continue the definitions:

17. GM_5 is the largest maximum difference in millimeters between any two consecutive five-minute readings of m beginning anywhere between t_{0-5} and t_{0+5}. Ex.: 18½ mm.

18. SM_5 is the smallest maximum difference in millimeters between any two consecutive five-minute readings of m beginning anywhere between t_{0-5} and t_{0+5}. Ex.: 17½ mm.

19. t_{GM_5} represents the times in minutes between which GM_5 occurs. Ex.: 10-15 and 15-20 min.

20. t_{SM_5} represents the times in minutes between which SM_5 occurs. Ex.: 10-15 and 15-20 min.

21. I_{GM_5} represents an index range calculated by dividing the values of m between which GM_5 occurs by their corresponding times multiplied by 3, since 3 mm. are equivalent to one minute. Ex.: 0.31 to 0.8.

22. I_{SM_5} represents an index range calculated by dividing the values of m between which SM_5 occurs by their corresponding times multiplied by 3, since 3 mm. are equivalent to one minute. Ex.: 0.27 to 0.72.

23. $\angle_{GM_5}$ is the angle opposite the horizontal side of a right triangle having GM_5 as the perpendicular side and 15 mm. (five minutes) as the horizontal side. Ex.: 39° 10′.

24. $\angle_{SM_5}$ is the angle opposite the horizontal side of a right triangle having SM_5 as the perpendicular side and 15 mm. as the horizontal side. Ex.: 40° 40′.

25. $\overline{M}_5$ is the mean of the M_5 calculations from t_0–t_{0+4}. Ex.: 18.1 mm.

26. $\angle_{\overline{M}_5}$ is the angle opposite the horizontal side of a right triangle having $\overline{M}_5$ as the perpendicular side and 15 mm. (five minutes) as the horizontal side. Ex.: 39° 50′.

27. V% is the centrifuged volume per cent of cellular material.

A chart of measurements of examples can now be presented:

	V%	em	t_{em}	I_{em}	$\angle^s{}_g$
Ex. 1.	36–	21 mm.	13.8 min.	0.51	39° 20′
Ex. 2.	31½	34 mm.	10.0 min.	1.10	18° 10′

	M_1	t_{M_1}	I_{M_1}	$\angle^s{}_{M_1}$
Ex. 1.	4.5 mm.	13-14 min.	0.45-0.52	33° 50′
Ex. 2.	9 mm.	9-10 min.	0.93-1.13	18° 20′

	M_5	t_{M_5}	I_{M_5}	$\angle^s{}_{M_5}$
Ex. 1.	17.5 mm.	10-15 min. 15-20 min.	0.27-0.57 0.57-0.72	40° 40′
Ex. 2.	31 mm.	5-10 min.	0.20-1.13	26° 00′

	GM_5	t_{GM_5}	I_{GM_5}	$\angle^s{}_{GM_5}$
Ex. 1.	18.5 mm.	10-15 min. 15-20 min.	0.31-0.80	39° 10′
Ex. 2.	34 mm.	5-10 min. 10-15 min.	0.23-1.57	24° 10′

	SM_5	t_{SM_5}	I_{SM_5}	$\angle^s{}_{SM_5}$
Ex. 1.	17.5 mm.	10-15 min. 15-20 min.	0.27-0.57 0.57-0.72	40° 40′
Ex. 2.	29 mm.	5-10 min. 10-15 min.	0.83-1.80	27° 30′

	$\overline{M}_5$	$\angle^s{}_{\overline{M}_5}$
Ex. 1.	18.1	39° 50′
Ex. 2.	32.0	25° 20′

COMPARISONS

Several features of the foregoing measurements of examples are quite noticeable:

1. Estimations of em are in all probability quite accurate because t_{em} in each case lies between the limits of t_{M_1} and also t_{M_5}, t_{GM_5} and t_{SM_5}.

2. Since the above is true, calculations of I_{em} are expected to lie and lie within the ranges of I_{M_1}, I_{M_5}, I_{GM_5}, and I_{SM_5}.

3. The nearer t_{em} approaches either of the limits of t_{M_1}, etc., the closer I_{em} approaches the corresponding limit of I_{M_1}, etc. Such would be expected.

4. $\angle^s_g$ are less than $\angle^s_{M_1}$, $\angle^s_{M_5}$, $\angle^s_{GM_5}$, $\angle^s_{SM_5}$, and $\angle^s_{\overline{M}_5}$ and upon examination of Fig. 5 rightfully should be.

When one considers that B′ represents in reality not a point on a straight line between A and C, but the point of change of a shallow sigmoid curve between A and C, then one will realize that the least angle between curve AB′ and a perpendicular line from B′ to AA′ will be less than angle ACA′. This also applies to estimates of angles derived from M_5, GM_5, SM_5, and $\overline{M}_5$. On first thought one would say then that the nearer the angles estimated from the differences between interval readings of m approach $\angle^s_g$, the more accurate they are. One might also say that the longer the interval between readings of m, the more deceiving will be the plotted curve and the more inaccurate the estimation of angles, maximum falls, indexes, etc.

Since smaller measurements are generally accompanied by larger possible errors, M_1 is thought to be accompanied by greater possible error than measurements of M_5. To illustrate this, consider the effect on M_1 and M_5 that a fifteen-second variation in t_0 will cause. Fifteen seconds is ¼ of one minute but only $\frac{1}{20}$ of five minutes. Also consider the effect on M_1 and M_5 that 0.5 mm. variation will cause. In example 1, 0.5 mm. is ⅑ of 4.5 mm. but only $\frac{1}{35}$ of 17.5 mm. Is it unreasonable to assume then that M_1 is subject to greater variations than M_5 and t_{M_5} in actual measurement? Also is it unreasonable to assume that errors in measurement of M_1 will produce greater error in estimation of $\angle_{M_1}$ than error in measurement of M_5 will produce error in estimation of $\angle_{M_5}$? Is it correct to assume then that, although $\angle_{M_1}$ and $\angle_{GM_5}$ more closely approach $\angle_g$, they are more susceptible to error as estimates of $\angle_g$ than $\angle_{M_5}$? In final analysis $\angle_{GM_5}$ is not derived from the maximum five-minute falls where measurement is started at t_0 but at times other than t_0, sometimes even at t_{0+4}. The same applies to $\angle^s_{SM_5}$ and $\angle^s_{\overline{M}_5}$. Their proximity to $\angle_g$ does not necessarily denote the degree of their accuracy.

Would it be in error to assume that if measurements of M_5 and $\angle^s_{M_5}$ on replicate aliquots are possible with a fair degree of accuracy these would at least roughly represent the separation of formed and fluid elements of the blood?

Evidence is convincing to me at least that the best estimate of separation is the continuous measurement of the line of demarcation between cells and plasma with estimation of the point of change in the resulting sigmoid curve. It is felt that if measurements of the point of change are to express even partially the important aspects of the curve they must include the time at which this point of change occurs (t_{em}), the amount of separation before the point of change takes place (em), and the rate of speed of separation immediately before the point of change (expressed as $\angle_g$).

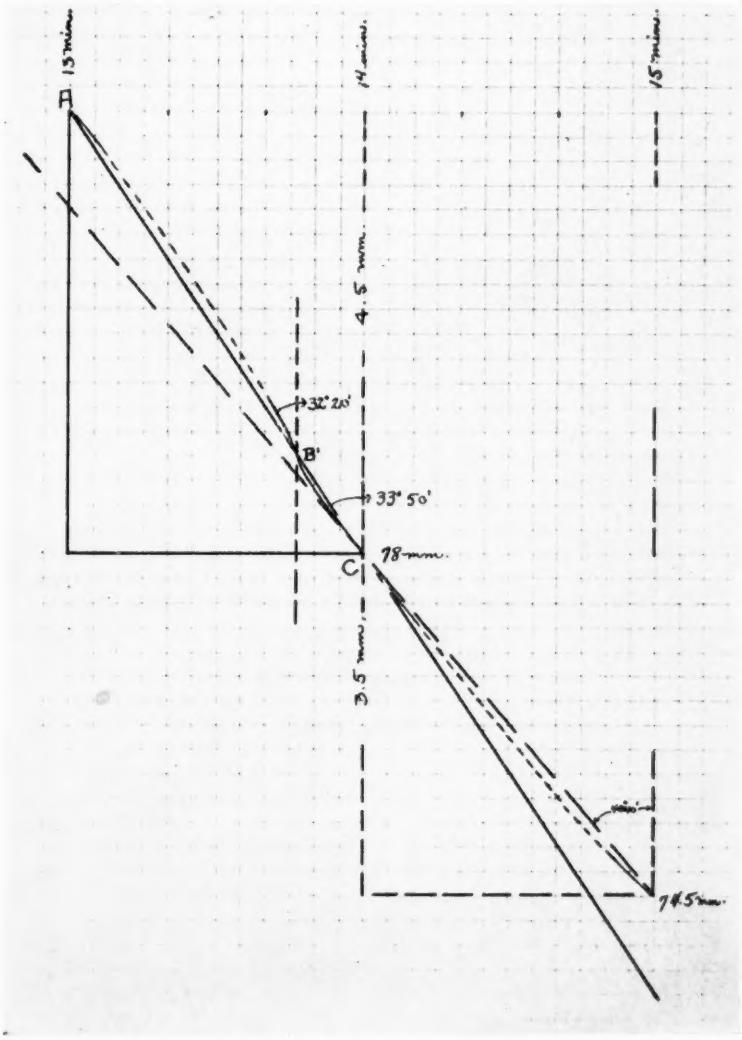


Fig. 5.—Ex. 1 of Fig. 3—graph of area near B′ (enlarged).

However, with practitioners, simplicity of technique is the keynote and often a certain degree of accuracy must be sacrificed for practicability. In view of this *a more practical technique* is devised, results of which will be reported in many instances along with results of the photographic technique and in many instances alone.

With this more practical "field" technique the blood sample can be drawn and divided in the same manner as described under the photographic technique. The vials are allowed to remain at room temperature until settled, are agitated as before, and the Wintrobe tubes are filled as previously described. However,

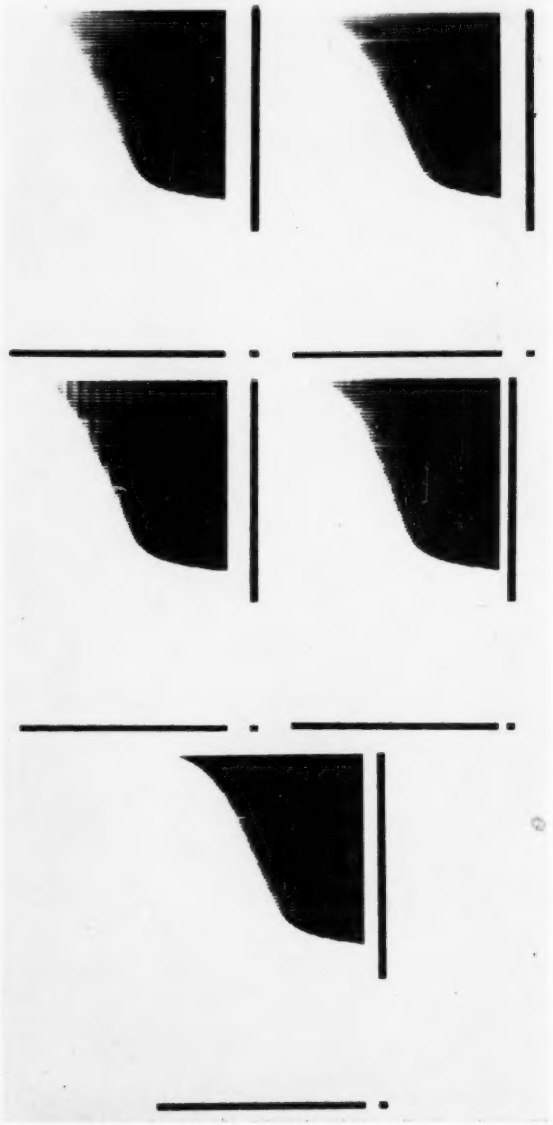


Fig. 6.—Evidence of the reproducibility of sedimentation curves as obtained from five aliquots of the same sample of blood.

instead of being put into the photographic recorder each tube is placed into the perpendicular slots of the previously described rack, and the line of demarcation between cells and plasma read five minutes after filling and at five-minute intervals thereafter until maximum settling is passed. The greatest difference in millimeters between any two consecutive five-minute readings is recorded. t_{M_5} and $\angle_{M_5}$ are determined. These three measurements can be considered as locat-

ing quite roughly, to be sure, the point of change between increasing and decreasing rate of descent of the line of demarcation between cells and plasma and the speed of separation.

REPRODUCIBILITY

Now it seems logical to test whether several aliquots of the same sample of blood will yield comparable results.

The reproducibility of the photographic technique is demonstrated by Fig. 6, representing curves of sedimentation in Wintrobe tubes filled from five 10 c.c. aliquots of the same sample of blood. Data from these are as follows:

Ex. No.	em	t_{em}	I_{em}	$\angle^{s}_{g}$	V%
1	29 mm.	9.2 min.	1.05	22° 50′	35.3
2	29 mm.	10.0 min.	0.97	22° 10′	35.3
3	29 mm.	9.0 min.	1.07	22° 30′	35.5
4	30 mm.	9.2 min.	1.09	22° 00′	36
5	30 mm.	10.0 min.	1.0	21° 30′	35.7
Mean	29.4 mm.	9.48 min.		22° 24′	35.6

Ex. No.	M_5	t_{M_5}	I_{M_5}	$\angle^{s}_{M_5}$
1	27 mm.	5-10 min.	0.53-1.17	29° 30′
2	25 mm.	5-10 min.	0.47-1.07	31° 20′
3	27 mm.	5-10 min.	0.67-1.23	29° 30′
4	28 mm.	5-10 min.	0.47-1.17	28° 30′
5	27 mm.	5-10 min.	0.27-1.03	29° 30′
Mean	26.8 mm.	5-10 min.		30° 04′

Since the determinants of I_{em} are em and t_{em}, I_{em} is thought superfluous. In addition I_{em} may be deceiving, since it expresses a ratio and could represent other values of em and t_{em} than those actually measured; for instance, an I_{em} of 1.0 might represent, as in Ex. 5, an em of 30 mm. and a t_{em} of ten minutes (30 mm.), but it might also represent an em of 15 mm. and a t_{em} of five minutes (15 mm.) or any other combination where em in millimeters is equal to t_{em} in millimeters. For this reason its use is dropped. Since I_{M_5} merely places rough limits to I_{em}, it too is dropped. An index of maximum "rise" ("fall" in this means of expression) over "run," which has been employed extensively in human techniques, has been neglected in this work chiefly because $\angle_{M_5}$ in reality is identical with this index. Since $\angle_g$ is felt to represent more accurately the actual "speed" of settling and since $\angle_g$ is best measured and expressed in terms of degrees and fractions thereof, for comparative purposes "speed" in the "field" technique is designated in this same terminology, keeping in mind, of course, that $\angle_g$ must be less than $\angle_{M_5}$.

Agreement in repetition of the "field" technique can be demonstrated by the following examples derived from measurements with Wintrobe tubes filled from five 10 c.c. aliquots of the same sample of blood:

Aliquot No.	M_5	t_{M_5}	$\angle^{s}_{M_5}$	V%
1	24 mm.	15-20 min.	32° 20′	34.5
2	23 mm.	15-20 min.	33° 30′	34.5+
3	24 mm.	15-20 min.	32° 20′	35.0-
4	25 mm.	15-20 min.	31° 20′	34.0+
5	25 mm.	15-20 min.	31° 20′	34.5+

Occasionally one will observe very close figures of M_5, $\angle^s M_5$, and V%, but $^t M_5$ will vary as in this example:

Aliquot No.	M_5	$^t M_5$	$\angle^s M_5$	V%
1	19 mm.	15-20 min.	38° 50′	41.0
2	20 mm.	15-20 min.	37° 20′	41.0-
3	19 mm.	20-25 min.	38° 50′	41.0+
4	18 mm.	20-25 min.	40° 10′	41.0-
5	18 mm.	20-25 min.	40° 10′	41.0+

This is not thought to be a serious error and the apparent reasons for such will be discussed in a future article under the heading of agitation. Further evidence of the reproducibility of the "field" technique will be observed as one continues through the remainder of this series.

In the next two publications of this series technical sources of variation will be demonstrated and discussed.

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OBJECTIVE METHOD FOR DEMONSTRATION OF LOCAL RIGIDITY OF THE ABDOMINAL WALL*

Alexander Baron, M.D., and Julius G. Baron, M.D.
Chicago, Ill.

LOCAL rigidity of the abdominal wall is a well-known symptom of some abdominal diseases. This phenomenon is commonly thought to be due to the increase in tension of some abdominal muscles and is detected by comparative palpation of symmetrical parts of the abdominal wall. However, the tactile sense is not reliable; even experienced observers in palpating the abdominal wall may get contradictory results. The assumption that local rigidity is caused by differences between tensions of both sides of the abdominal wall has been challenged by Vaughan.[1] According to him, such differences can be observed only if the two parts are palpated one after the other. Vaughan asserts that palpation of the diseased side elicits a contraction of the abdominal muscles and this contraction occurs equally on both sides. Thus, if both sides are being palpated simultaneously by both hands, there is no difference observed except in

*From the Department of Biology and the Department of Physics, De Paul University, Chicago, and the Alexian Brothers Hospital, Chicago.
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the presence of a palpable mass on the affected side. Wrork,[2] too, questions the occurrence of differences between the tensions of the two sides of the abdominal wall. However, at least the possibility of such differences is shown by the example of the diaphragm. In inflammatory conditions of the abdomen, when the lesion is close to one side of the diaphragm, immobilization of this side of the diaphragm in high position can often be observed by fluoroscopy. Further, in cases of acute appendicitis, Sale[3] found that the right diaphragm was, as a rule, flatter than the left.

To compare the resistance of symmetrical parts of the abdominal wall in an *objective* manner, equal forces acting in symmetrical directions and upon equal areas should be applied, and the depth of the depressions measured. However, the movements of the abdominal wall during breathing interfere with the making of such measurements. Evidently the measurements should be made in a respiratory pause and on both sides in the same phase of respiration. Kelley[4] described an apparatus which he used to make such measurements. However, in Kelley's method the direction of the force cannot be established accurately, and no provisions are set forth for making the measurements in the same respiratory phase. Wrork[2] made similar measurements in normal and pathologic cases with an apparatus in which a pair of weights are used to exert forces upon symmetrical parts of the abdominal wall. The whole apparatus rests on the abdominal wall. As the result of his measurements, Wrork makes the statement that no differences between the tensions of the two sides of the abdominal wall occur. However, if the forces exerted by the cylindrical weights are to be definite, the longitudinal axes of the weights must be kept exactly vertical. Wrork sets the crossbar of the apparatus in a horizontal position. But the position of the whole apparatus is subject to changes caused by the respiratory movements of the abdominal wall because it seems hardly possible that the subject could hold his breath during the whole time of setting the apparatus by a spirit level and making the measurements. Thus Wrork's measurements do not seem to prove his assertion that no differences occur between the tensions of the two sides of the abdominal wall.

To demonstrate local rigidities of the abdominal wall in an objective way a new method has been developed which is based upon the detection of differences between the respiratory movements of symmetrical parts of the abdominal wall. In this way, instead of being a disturbing factor, the respiratory displacement of the abdominal wall becomes the object of the measurement. In other words, in making the measurements the force originating from the contraction of the diaphragm is used rather than external forces. The contracting diaphragm acts like a piston producing compression, thus increasing the intra-abdominal pressure during inspiration. If the subject is in an exactly horizontal supine position, the pressure forces acting upon equal areas of symmetrical parts of the abdominal wall are equal and of symmetrical direction. The following discussion shows, namely, that the abdominal cavity can be considered as a closed container filled with a liquid, and that the conditions of equilibrium of this cavity and the pressure forces acting upon its surface are subject to the laws of hydrostatics.

VALIDITY OF THE LAW OF PASCAL FOR THE ABDOMINAL CAVITY

The law of Pascal states that if only surface forces are acting upon a liquid, the pressure is the same at every point and in every direction. True, the contents of the abdominal cavity are solids. However, a solid body which does not resist bending and is not acted upon by tensile forces behaves statically like a liquid and obeys Pascal's law. This is true for a considerable part of the abdominal organs (stomach, intestines, peritoneal folds) which, when put into an open container, assume its form and present a horizontal surface. The other group of the abdominal organs are solids in every respect and, therefore, do not obey the laws of hydrostatics in their interior. Their presence, however, does not affect the validity of the law of Pascal for the abdominal cavity just as the presence of solid bodies would be of no consequence upon the validity of the same law for a real fluid. Upon the contents of the abdominal cavity, in addition to surface forces originating from the tension of the abdominal walls, a mass force—gravity—is acting. The effect of the latter is shown by pressure differences between various levels of the cavity, all points of the same level having the same pressure. Since differences in specific gravity of the solid organs and of their surroundings are negligible, these organs float freely, hindered in their movements only by peritoneal bands. Under ordinary conditions the bands are loose and the solid organs are acted upon solely by the intra-abdominal pressure. Naturally, these bodies establish equilibrium by exerting exactly the same pressure on their surroundings. Thus, whether or not the abdominal walls are in direct contact with solid organs, no forces other than originating from the abdominal pressure are acting on their internal surface. There are only two exceptions: First, the anterior and posterior abdominal walls may be forced apart by a solid organ during the whole period of respiration or, more often, during deep expiration only. In this case a local increase in pressure will result while the organ itself undergoes elastic deformation. The other exception concerns gas-filled intestinal loops. Such a loop has a mean specific gravity considerably less than that of its surroundings, and thus it is acted upon by a buoyant force. Hence the loop tends to occupy the highest possible position, either stretching the corresponding bands, or protruding the abdominal wall. With these two exceptions, which will be discussed later, the equilibrium positions of the different parts of the abdominal wall in a given position of the subject are determined by the elastic properties of this wall and by the intra-abdominal pressure. They are not influenced by the position of the abdominal organs, such as adjacency to the abdominal wall. The slow respiratory movements of the abdominal wall can be considered as consisting of a continuous series of equilibrium positions. Thus, with the subject in an exactly horizontal supine position, simultaneous respiratory amplitudes of symmetrical parts of the abdominal wall must be equal if the structures of the two sides of the abdominal wall are in every respect alike. On the other hand, any unilateral change may create a difference between simultaneous respiratory amplitudes of the two sides.

APPARATUS FOR THE OBSERVATION OF DIFFERENCES BETWEEN RESPIRATORY AMPLITUDES

The method to be described involves the use of an apparatus somewhat resembling a large caliper. It has two aluminum legs (about 15 cm. long) connected at the top by a nearly frictionless hinge which allows them to move freely in only one plane. The length of the legs can be increased to about 30 cm. by screwing on extensions. The end of the legs and of the extensions are pointed. A sharp-pointed steel index rod, about 2 cm. long, is attached to the hinge perpendicularly to the plane of the legs. The weight of the apparatus with extensions is 40 Gm. For the examination the subject is placed in a reclining position so that his frontal plane is horizontal, the knees being slightly bent and supported by pillows. The parts to be compared are covered by small pieces of

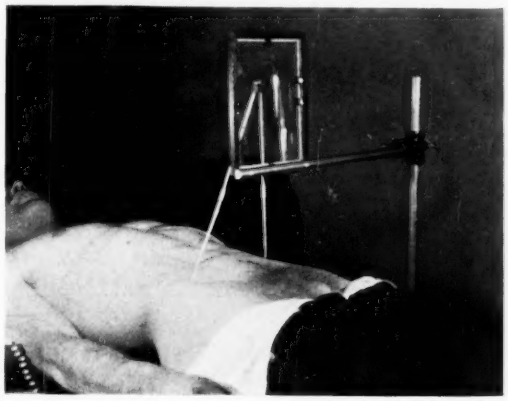

Fig. 1.

adhesive tape and the points of the legs are placed thereon. To the table on which the subject is lying a glass plate, 8 by 20 cm., with reference line in the middle, is attached in such a way that the line is vertical. The instrument is then leaned against the plate, the point of the index rod near the reference line (Fig. 1). The angle between the plate and the plane of the legs should be about 10°, and symmetrical points of the legs should be at equal distance from the glass plate. If the distance between the points examined is about 6 to 7 cm. then the shorter legs should be used; if the distance is about 12 to 15 cm. the extensions are required; the angle between the two legs should be approximately 25°. There is little friction between the glass plate and the index point because the weight of the instrument has only a small component perpendicular to the glass plate. During breathing the point of the index rod moves along the glass plate. If the points of the legs are moved through the same distance along vertical lines, then the point of the index rod will also describe a vertical line. On the other hand, if the respiratory amplitudes of the points compared are not the same, the point of the index rod will shift to the one side or the other. Therefore, such a lateral shift signifies an existing difference between the tensions of the two sides of the abdominal wall. In reality the various parts of the abdominal wall move during respiration laterally as well as vertically. However,

these lateral displacements are small compared to the vertical ones, and thus, as a simple geometrical construction shows, only the vertical differences need be considered.

The examination can be made most conveniently when the breathing is purely abdominal and the movements of the abdominal wall are not too small. Most of the subjects may easily be induced to breathe in this manner, particularly so if they are told to breathe with an open mouth. But whatever the type of breathing may be, if the movements of the abdominal wall are large enough, the examination gives clear-cut results. If the intra-abdominal pressure increases, that is, if the abdominal wall moves outward, the index point moves toward the more tense side; if the intra-abdominal pressure decreases, that is, if the abdominal wall moves inward, the shift of the point is to the less tense side; and no sidewise shift signifies the absence of a difference in tension. Since the difference between the respiratory amplitudes changes with various depths of breathing, one should observe the angle between the vertical and the line described by the index point rather than the lateral shift of the index point. Other things being equal, the greater this angle, the greater the difference of tensions of the two sides. A graph of the path of the index point is recorded on the glass plate if it is coated by lampblack. In cases showing differences in tension of the two sides, this curve usually consists of three parts: a lower vertical part, corresponding to deep expiration; a slightly S-shaped part which makes an angle with the vertical, and corresponds to the middle phase of respiration; and an upper vertical part, corresponding to deep inspiration. Experiments made with an unevenly stretched rubber membrane showed that an inanimate structure can behave similarly. If the anterior and posterior abdominal walls are forced apart by a solid organ, the corresponding part of the anterior abdominal wall will not move during respiration. In protrusion of one side of the abdominal wall caused by a gas-filled intestinal loop, the difference in level of the two sides is diminished both in deep inspiration and in deep expiration. Thus the index point will describe a shallow C-shaped curve open to the protruded side. In practical work it is not necessary to record the curve traced by the point of the caliper. It is easy to recognize the shape of the curve and to estimate the angle formed by the middle portion of the curve and the vertical line with sufficient accuracy.

TABLE I

CASE	DEVIATION		
	UPPER RECTUS	LOWER RECTUS	LATERAL
1	–	+ L	+ L
2	–	+ L	+ + L
3	–	+ + L	+ L
4	+ R	+ L	+ L
5	+ L	–	–
6	+ R	+ L	+ L

+ = Slight deviation (up to 15°).
+ + = Marked deviation (between 15° and 30°).
R = to right. L = to left.

Fifty male college students, apparently in good health, were examined by the method described. The subject was placed on a table so that his frontal plane was exactly horizontal. Three pairs of symmetrical points of the ab-

dominal wall were used. One pair between the xyphoid process and the navel, 7 cm. apart (upper rectus points), one pair in the level of the navel, 7 cm. apart (lower rectus points), and a third pair 3 cm. below the navel, 14 cm. apart (lateral points). In 28 cases the point of the caliper moved vertically in all three positions of the caliper, showing that there was no difference between the tensions of the two sides of the abdominal wall. Insignificant deviations in some positions of the caliper (less than 10°) were observed in 16 cases. The results obtained in the remaining 6 cases are shown by Table I.

Observations were also made in a series of patients having abdominal complaints. Deviations greater than 15° (up to 60°) were observed in cases of acute appendicitis, cholecystitis, duodenal ulcer, splenic tumor, and also in spastic conditions of the gastrointestinal tract.

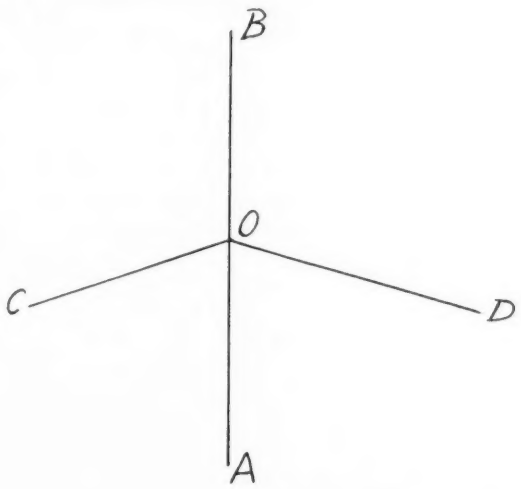

Fig. 2.

These findings were not always in accordance with palpatory findings. This is probably due to the fact that an increase of tension on the side of the pathologic condition may be elicited by palpation itself. Though the observations made in pathologic cases seem to show that the caliper method may have definite clinical value, more extensive observations must be made before the practical importance of this method can be properly established.

COMMENT

Obviously, the observed differences between the respiratory amplitudes of symmetrical parts of the abdominal wall are caused by differences between the structures of the two sides of this wall. These differences may be persistent, either acquired or congenital. More interesting from the standpoint of abdominal pathology are the more or less transitory differences caused by unilateral changes in tone* of the muscles of the abdominal wall. If the tone of one of the rectus muscles increases, the tension on the same side becomes greater and

*The *tone* of a muscle is considered to depend upon the percentage of the fibers which are in a state of contraction. If the muscle tone is kept constant, then in a given muscle the *tension* (which is a purely physical conception) is exactly determined by the length of the muscle.

the respiratory amplitude of every point of this side is smaller than that of the corresponding points of the other side. If the tone of the lateral muscles (transversus and obliqui) of one side increases, an increase of the tension of both sides results. However, the following example shows that the tension of the side having the greater tone will be larger. If two strings AB and CD are tied together at O and stretched in directions, as shown in Fig. 2, the tension along AO will be greater than along BO because O is pulled toward B by COD. Thus a difference of tension is present between two parts of a straight string. In the same way different tensions may be present at various points of the stretched abdominal wall or in various directions about a single point. The difference between the respiratory amplitude of the upper parts of the right and left rectus muscles and the difference between the respiratory amplitude of the lower parts can have opposite signs (Cases 4 and 6, Table I) only if the tensions in various segments of the rectus muscle are different. The fact that a major portion of the muscle fibers originates or ends at the tendinous intersections of this muscle makes it possible that considerable differences in tone be present at the two sides of such an intersection. An increase in tone of a certain part of the rectus muscle causes the interior points of this muscle to move in the direction of this part. In this way the fibers of the lateral muscles are pulled out of position and, therefore, exert an oblique pull on the part of the rectus muscle which increased its tone. A difference between the tensions of parts of the rectus muscle will thus result.

SUMMARY

The amplitudes of the respiratory movements of the abdominal wall depend —other things being equal—upon the tension of this wall. By comparing the respiratory amplitudes of symmetrical parts of the abdominal wall while the subject is in an exactly horizontal position, differences between the tensions of the two sides can be demonstrated in an objective manner by using a caliper-like instrument. No significant differences between the tensions of the two sides were observed in 44 out of 50 healthy subjects. In the remaining 6 cases there were marked differences. Differences of a still greater magnitude were observed in various pathologic conditions of the abdominal organs. These observations seem to prove the reality of the palpatory findings of local rigidities of the abdominal wall.

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RUBBER PERINEUM*

ARTHUR C. STIRLING, M.D., ROCHESTER, N. Y.

FOR the medical student, when he reaches his course in obstetrics, few new experiences offer so much confusion as when he is first called upon to repair a perineal tear or an episiotomy following a delivery. This is so due to the considerable distortion of structures and the abundance of blood in the field.

It is of value for the student to know how to do this relatively simple and common operation before he actually practices upon a patient; the patient profits in his knowing by having a more comfortable and a more perfect repair; the instructor profits by saving of time and disposition at the time of the student's first attempt on a patient; and the student profits by a sense of knowledge and pride in his exactness of technique. Heretofore, to my knowledge, there have been no methods to facilitate the instruction and the practice of this operative procedure other than on the patient.

The device described and illustrated here is of simple construction, is durable enough for about twenty-five practice trials, and may be easily renewed. The student who is quick to learn a new technique can learn to repair an episiotomy in three trials. The method of repair employed will differ with the medical school or with the individual giving the instructions. It is believed that most ordinary methods may be demonstrated accurately and practiced on this model. The main purpose of the device is to teach the student how to lay the sutures in reference to mucosa, and deep and cutaneous tissues, in order to attain a certain degree of perfection before actually repairing a patient.

The essential feature of the device consists of an air foam type of sponge rubber of such a size that an incision simulating an episiotomy may be made across one edge. The consistency is such that the usual curved round surgical needle may be used without tearing the rubber. An oblong piece of bath sponge purchased for ten cents may be cut into two suitable pieces. The sponge is held firmly to a formed base by a system of prongs and spring arms. The base may be made of wood or cast iron with a thin rubber base to prevent slipping on the table top. The spring arms also serve to separate the edges of the incision, exposing what may be termed the "deeper fascial layers"—thus permitting the student to lay deep figure-of-eight or single interrupted sutures. When worn, the sponge may be easily replaced. There is also mounted on the base an upright peg for holding a spool of moderately heavy cotton thread with which the repair is made.

It has been found satisfactory to use a Bartlet Mayo tapered point curved needle. The needle holder may be a discarded one from the operating room or

*From Department of Obstetrics and Gynecology, Strong Memorial Hospital, Rochester. Received for publication, March 31, 1942.

any suitable clamp. A pair of scissors or a razor blade completes the essential equipment. With this device one may place sutures simulating deep, mucosal, and subcutaneous stitches with accuracy. By replacing the episiotomy sponge with one in which a simple linear incision has been made, the student may practice ordinary skin closures.

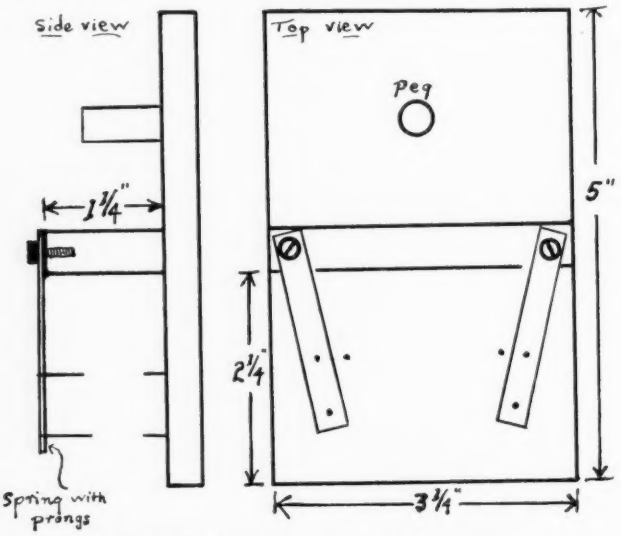

Fig. 1.—Rubber perineum. Scale drawing.

A. *B.* *C.*

Fig. 2.—Rubber perineum. Photographs illustrating the laying of sutures in the vaginal mucosa, deep structures, and finally the subcutaneous tissues in the repair of a medial episiotomy. Mediolateral episiotomies and tears are very similarly repaired.

Regardless of the amount of material available, this rubber perineum will be of great assistance to the student before he actually attempts a repair on a patient.

Acknowledgment to F. F. Baker, M.D., and Jane Stirling for the photographs.

A METHOD FOR EXPOSING CULTURES TO CARBON DIOXIDE OR ANAEROBIC ATMOSPHERES IN PETRI DISHES*

EDWARD J. FOLEY, NOTRE DAME, IND.

THE necessity of incubating a few Petri dish cultures under anaerobic conditions or under carbon dioxide tension frequently arises in most bacteriologic laboratories. The need for individual units has been recognized for many years, and two devices of particular merit have been described—the McLeod apparatus[1] and the Spray dish.[2] Both take advantage of the principle of absorption of oxygen by mixtures of alkali and pyrogallic acid. Two compartments are provided in the bottom chamber by means of a built-in barrier, and alkali and pyrogallic acid are placed in the separate compartments. The half of the Petri dish which contains the inoculated agar medium is inverted over the bottom chamber, and the joints are sealed. After sealing, the dish is tipped, effecting a mixture of the solutions.

The Spray dish has been used successfully for growing cultures in carbon dioxide atmospheres produced by mixing sulfuric acid and potassium or sodium bicarbonate by Joyner and Jones.[3]

We have found that the Spray dish technique for anaerobic or carbon dioxide cultivations can be made without the use of specially designed apparatus. The only materials required, in addition to the reagents, are modeling clay and 100 by 15 mm. and 75 by 10 mm. Petri dishes.

Anaerobic cultures are prepared as follows: A rather generous roll of modeling clay is fashioned and placed over the lip of the bottom half of a 100 by 15 mm. Petri dish. A shallow bottom half of a 75 by 10 mm. Petri dish is selected and placed open side up in the larger dish. Four cubic centimeters of a 20 per cent solution of pyrogallic acid solution are run into the bottom of the large dish and 5 c.c. of a 20 per cent sodium hydroxide solution (or 7 c.c. of a 20 per cent sodium carbonate) into the small one. The culture is made in a 100 by 15 mm. Petri dish by pouring inoculated cool agar, or by streaking a hardened agar plate followed by pouring a shallow layer of cooled agar over the surface. The tendency of colonies to coalesce is reduced in such cultures. After the medium has hardened, the half containing the culture is inverted and pressed down firmly into the clay roll. Complete sealing is achieved by flattening the excess remaining on the outside surfaces where the lips of the plates approximate. The reagents are mixed by careful tilting and rotating. The smaller plate slides to the edge of the large one mixing the contents.

An approximate 20 per cent carbon dioxide atmosphere is prepared in the same way, except that 2 c.c. of sulfuric acid solution (1 c.c. of concentrated sul-

*From the Laboratories of Bacteriology, University of Notre Dame.
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furic acid in 29 c.c. of distilled water) and 2 c.c. of sterile 1 per cent sodium carbonate solution are used.

Effective anaerobiosis produced by this means has been demonstrated consistently to last for ten days, this being the limit of the observation period. Both methylene blue contained in dextrose beef infusion agar and fresh rabbit blood mixed in agar were used as indicators.

Two per cent agar in thioglycollate medium (Baltimore Biological Laboratories) has been used to advantage in this device. More rapid and perhaps more efficient, anaerobiosis is obtained with this medium under the influence of alkaline pyrogallic acid than occurs with ordinary agar.

SUMMARY

A simple method is described by which individual solid media cultures in Petri dishes can be incubated under carbon dioxide tension or under anaerobic conditions.

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METHOD FOR DETERMINING THE TOXICITY OF ANTISEPTICS AS MEASURED BY THE DESTRUCTION OF HUMAN LEUCOCYTES*

Henry Welch, Ph.D., Glenn G. Slocum, Ph.D., and Albert C. Hunter, Ph.D.
Washington, D. C.

A METHOD has been presented previously[1] for determining the primary toxicity of antiseptic solutions, as measured by the loss of the phagocytic function of the leucocyte. By comparing loss of phagocytic activity with the bactericidal action of a compound, it is possible to compute a "toxicity index," which is defined as the greatest dilution of the antiseptic that completely inhibits phagocytosis, divided by the greatest dilution which kills the test organism. Later work[2] indicated that the toxic effect of germicides, when tested by this method on human or guinea pig blood as a tissue, was, in most instances, directed against the humoral elements, in contrast to the cellular elements. However, with mercuric chloride and certain other compounds evidence was presented[3] to show that such solutions, when tested by their effect on hemolytic complement, exhibited definite toxicity but, in contrast to iodine, prevented phagocytosis not by an actual destruction of the humoral or cellular elements involved but by a "blocking" of the effective mechanisms. It was apparent in this type of test that concentrations of antiseptic, which brought about cessation of phagocytosis, were not sufficiently great to cause actual destruction (irreversible loss of function) of the cells.

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It was further apparent from our studies that certain components of the humoral system were sensitive to the toxic action of the majority of antiseptics tested and were thus destroyed by their action, while in those compounds which reacted like mercuric chloride, a "blocking" effect resulted which prevented phagocytosis. This latter effect, however, could be counteracted by dilution of the mixture of plasma and chemical. By testing toxic activity on "naked" cells[2] (blood centrifuged free of plasma), it was shown that greater concentrations of antiseptic were necessary to inactivate such cells than were found necessary to stop their activity when the toxic effect was directed against the humoral system, an essential cog in the phenomenon of phagocytosis.

Through studies of a variety of techniques for determining the effect of antiseptics on the human cell, it soon became apparent that the use of cells free of plasma was neither an efficient nor an appropriate method. Difficulties were encountered when attempts were made to produce a homogeneous cell suspension which would give uniform results when mixed with increasing dilutions of the compound under test. It appeared that relatively viscid cell suspensions failed to contact evenly the solution tested in the amounts used either through purely mechanical improper mixing during rotation or through absence of sufficient amounts of electrolytic solution in the mixture. For example, when mercuric chloride was tested on whole human blood, the toxic dilution was found to be 1:1,300, while on packed "naked" cells mixed with an equal volume of antiseptic dilution a toxic end point of 1:500 was obtained. Although a dilution of 1:600 or 1:700 showed marked phagocytic activity, dilutions of 1:800 nevertheless showed considerably less activity in spite of the lowered concentration of toxic substance. It was apparent that when the mercury salt was present in high concentrations, destruction of all cells was accomplished in spite of improper mixing of cells and test substance due to an excess of the chemical, while at lower more critical concentrations the effect of poor mixing of ingredients becomes obvious.

In the development of the method presented in these studies, consideration was given also to the fact that under the conditions of use in or on the human body antiseptics do not come into contact with naked cells but with serous exudates as well, and this material offers a certain amount of protection to the cell. Furthermore, it seemed particularly desirable to use whole blood, as a tissue, gauging the toxicity of the compound under test by its toxic effect on an individual cell of that tissue, since such a procedure approaches more closely the conditions met when antiseptics are used on other tissues of the body.

Accordingly, in the present investigation a method has been developed to determine the effect on the leucocytes directly, using whole blood as a tissue. Irreversible loss of phagocytic activity is taken as an indication of lethal action on the leucocyte.

TECHNIQUE OF TEST

The antiseptic dilutions to be tested are pipetted in 0.1 c.c. amounts to the bottom of test tubes measuring 100 by 13 mm. (Wassermann tubes). To the antiseptic is then added 0.4 c.c. of human blood which has been diluted 1:2 with 0.8 per cent citrated salt solution. (The blood is collected in sufficient 20 per

cent sodium citrate in salt solution to give a final concentration of approximately 0.77 per cent citrate. Usually 20 c.c. of blood are mixed with 0.8 c.c. of 20 per cent citrate.) The mixture of citrated blood and antiseptic is then rotated at 37° C. for ten minutes on a serum-leucocyte mixing machine[1] revolving at 4 r.p.m. As soon as the tubes are removed from the rotator, 2.0 c.c. of 0.8 per cent citrated salt solution are added immediately to each tube to stop further action of the antiseptic. The tubes are shaken vigorously, centrifuged at 2,500 r.p.m. for eight minutes, and the supernatant fluid is removed and discarded. Care should be taken not to disturb the buffy coat of leucocytes usually stratified on the red blood cells when removing the supernatant fluid. The cells which have been in contact with the antiseptic under test for a period of ten minutes are now tested for phagocytic activity by the addition of 0.2 c.c. of antigen and 0.1 c.c. of fresh human plasma. The antigen consists of a standardized suspension of artificially opsonized staphylococci, the method of preparation of which has been described[1] previously. Following addition of antigen and plasma in that order, the mixture is rotated on the serum-leucocyte mixing machine again at 37° C. for twenty minutes. Blood smears are prepared, stained for one minute with 1 c.c. of 0.5 per cent methylene blue in absolute alcohol, following which 1 c.c. of distilled water, buffered at pH 7.2, is added and staining continued for four minutes longer. The degree of phagocytosis is then determined.

TABLE I

METHOD I To show the toxicity of antiseptics as measured by inhibition of mechanism of phagocytosis	METHOD II To show the toxicity of antiseptics as measured by death of the cell (irreversible loss of function)
1. Mixture: 0.1 c.c. antiseptic 0.2 c.c. antigen 0.2 c.c. human blood	1. Mixture: 0.1 c.c. antiseptic 0.2 c.c. citrated salt solution 0.2 c.c. human blood
2. Rotated on mixing machine for thirty minutes	2. Rotated on mixing machine for ten minutes
3. Smears prepared and stained with methylene blue for five minutes	3. Two cubic centimeters of citrated salt solution added and mixture centrifuged at 2,500 r.p.m. for eight minutes
4. Degree of phagocytosis determined	4. Supernatant fluid removed and activity of cells tested by the addition of: 0.2 c.c. antigen 0.1 c.c. fresh human plasma
	5. Rotated on mixing machine for twenty minutes
	6. Smears prepared and stained with methylene blue for five minutes
	7. Degree of phagocytosis determined

As the concentration of antiseptic is increased, the amount of phagocytosis decreases until a concentration is reached at which no phagocytosis is observed. The degree of phagocytic activity is determined, and complete inhibition of phagocytosis is taken as an end point. The differences betweeen the original method,[1] which measured inhibition of the function of the leucocyte, and the method above, which determines that dilution of antiseptic lethal for the cell,

are shown briefly in Table I. By the use of the two methods described for determining relative toxicity, 26 germicides have been tested. Table II lists the 26 chemical preparations and, wherever possible, the dilution of the active ingredients commonly sold on the market or recommended for use, the dilutions of the active ingredients found toxic by each method, and the corresponding toxic dilutions of the commercial product or recommended concentration.

TABLE II

COMPARISON OF THE TOXICITY OF ANTISEPTICS FOR HUMAN TISSUE AS MEASURED BY INHIBITION OF MECHANISM OF PHAGOCYTOSIS AND DESTRUCTION OF THE CELL

ANTISEPTIC	ACTIVE INGREDIENT DILUTION IN COMMERCIAL PRODUCT	DILUTION OF ANTISEPTIC COMPLETELY INHIBITING PHAGOCYTOSIS			
		WHOLE BLOOD, INHIBITION OF MECHANISM OF PHAGOCYTOSIS (METHOD I)		WHOLE BLOOD, DESTRUCTION OF CELLS (METHOD II)	
		DILUTION OF ACTIVE INGREDIENT	DILUTION OF COMMERCIAL PRODUCT	DILUTION OF ACTIVE INGREDIENT	DILUTION OF COMMERCIAL PRODUCT
Acriflavine	1:1,000	1: 2,000	1: 2	1:1,200	1: 1.2
A (complex organic chlorine compound)	1:3,300	1: 5,000	1: 1.5	1:3,000	Not toxic
B, Tr. (pyridinium compound)	1: 200	1: 5,500	1:27.5	1:2,500	1:12.5
B_1, Aq. (pyridinium compound)	1:1,000	1: 3,000	1: 3	1:2,500	1: 2.5
Chloramine-T	1: 100	1: 700	1: 7	1: 200	1: 2
*Dodecylamine acetate		1:13,000		1:8,500	
†E-605		1: 900		1: 800	
†E-607		1: 1,000		1: 900	
†E-609		1: 3,500		1:3,000	
C (resorcinol derivative)	1:1,000	1: 5,000	1: 5	1:1,700	1: 1.7
Iodine tincture	1: 14.3	1: 600	1:40	1: 600	1:40
D, Tr. (organic Hg compound + cresol derivatives)	1: 500	1: 7,500	1:15	1:3,500	1: 7
Mercuric chloride		1: 1,300		1:1,300	
E (organic Hg compound - dye)	1: 50	1: 1,700	1:34	1: 60	1: 1.2
F, Tr. (organic Hg compound)	1:1,000	1:17,000	1:17	1:8,500	1: 8.5
G (phenyl mercury salt - acetate)		1: 1,900		1:1,500	
H (phenyl mercury salt - nitrate)	1:1,500	1: 3,000	1: 2	1:2,000	1: 1.33
I, Tr. (organic Hg - cresol compound)	1: 200	1: 5,400	1:27	1:3,000	1:15
Oxyquinoline sulfate		1: 700		1: 200	
Phenol		1: 400		1: 150	
Potassium mercuric iodide, aqueous	1:5,000	1: 1,100	Not toxic	1:1,100	Not toxic
Potassium mercuric iodide, 70% alcohol	1: 100	1: 2,000	1:20	1:1,500	1:15
Potassium permanganate		1: 750		1: 200	
Sodium ortho-phenylphenate		1: 900		1: 600	
J (cationic detergent)	1:2,000	1: 3,000	1: 1.5	1:2,500	1: 1.25
Zinc sulfocarbolate		1: 100		1: 20	

*This material was obtained from John H. Glynn, M.D. (Armour and Co.) and has been described in the J. Am. Chem. Soc. **63**: 1916, 1941; Ibid. **63**: 2576, 1941.

†The formulas for these compounds are given in J. Exper. Med. **74**: 612, 1941. In a previous publication (J. Immunol. **43**: January, 1942) an incorrect interpretation of the chemical make-up of E-605 and E-607 was given.

It will be noted that of the 26 germicides tested, 23 show lower toxicities when toxicity is measured by the direct action on the cells (Method II), while only three show equivalent toxicities by both methods. The marked difference in toxic effect, as measured by the two methods, is brought out particularly with compound "E," which has a toxic end point of 1:1,700 (Table II) when toxicity is measured by the loss of phagocytic function, while a toxic end point of

only 1:60 is obtained when destruction of the leucocyte is taken as an indication of toxic effect. Thus, by the latter method a dilution of only 1:1.2 of the commercial product (2 per cent solution) would represent the toxic dilution of this product, while by the former method a dilution of 1:34 of the commercial product would be toxic. It is of interest that the concentrations of this chemical inhibiting phagocytic function as compared to concentrations which destroy the cell are comparable to those concentrations of this compound which are bacteriostatic and bactericidal.

When destruction of the cell is taken as a measurement of the toxicity of a product such as compound "A," it will be noted that since a 1:3,000 dilution of the active ingredient destroys cells, and the product usually is marketed for use in a 1:3,300 dilution, this product would not be toxic for the cell at the dilution commonly used, although it will be noted that it interferes with the mechanism of phagocytosis at a dilution of 1:5,000. A similar result was obtained with an aqueous solution of potassium mercuric iodide, usually recommended in a 1:5,000 dilution of the active ingredient. As a matter of fact, it will be noted in Table II that besides compound "A" and aqueous potassium mercuric iodide, which are not toxic for the cell in commonly used dilutions, a number of the compounds tested are toxic only at relatively low dilutions of the commonly used strengths when toxicity is measured by direct action on the cell, as exemplified by acriflavine, chloramine-T, and compounds "B_1," "C," "E," "H," and "J."

DISCUSSION

The results obtained by the proposed method, where irreversible loss of function of a human cell is taken as evidence of the toxic activity of antiseptics, indicate that greater concentrations of the compounds tested are required than when reversible loss of function of these same cells is taken as evidence of toxic activity. In the former instance that concentration of antiseptic which destroys the activity of the cell is determined, while in the latter instance the concentration of antiseptic which destroys or interferes with some necessary component of the humoral system is determined.

Where evidence is desired to show the relative toxicity of a group of antiseptics either method may be useful. The method utilized depends upon whether interference with phagocytosis or destruction of tissue cells is the important consideration. In either case the compounds would all be tested under the same set of conditions.

The use of human blood as a tissue and the leucocyte as the test cell seem especially appropriate for measuring toxicity under practical conditions. Human blood is probably the most readily accessible tissue. It is uniform, constant in its pH, reasonably strongly buffered, and in the practical application of antiseptics is the most likely of all the specialized tissues to be encountered. It has been apparent to us in our studies that the leucocyte is relatively resistant to the action of toxic agents and compared to other tissue cells it is unique in its independent nature and its ability to survive for reasonably long periods of time though removed from its natural environment. The resistance of the leucocyte to antagonistic agents, including certain detergents, is rather remarkable and

deserves special comment. With certain dilutions of these compounds it was noted in many instances that where the red blood cells were completely hemolyzed and the plasma coagulated, the leucocytes continued to carry on their phagocytic function undiminished when fresh plasma was added to them. When the resistance of the leucocyte is compared to the resistance of most other body cells in situ, it is generally accepted that the latter are probably less affected by toxic compounds than the former. On the other hand, because of their interdependence on one another, individual tissue cells undoubtedly have less resistance than leucocytes to the action of toxic compounds. It is undoubtedly true, as well, that many tsisue cells in their natural habitat are destroyed by the toxic action of antiseptics, although such destruction is difficult to determine and becomes apparent only when such large numbers of cells die that the structure or continuity of the tissue is broken down. Therefore, the toxic effect of an antiseptic on the leucocyte may be taken as evidence of a similar if not greater effect on other individual tissue cells. Furthermore, the mechanism of phagocytosis is manifestly an important one in protection of the host against invading organisms, and since the leucocyte acts as one of the antibacterial agents of the body, chemical antiseptics should augment rather than reduce the activity of these cells.

The results obtained with tincture of iodine, mercuric chloride, and potassium mercuric iodide, all three of which showed the same toxic end point by both methods reported, deserve some comment. As previously reported[2] all three of these compounds had a lower toxic end point when tested on the naked cell than when tested on whole blood, using inhibition of the mechanism of phagocytosis as an end point. The differences in toxic end points of these compounds appear to be associated with the type of test used to demonstrate cell destruction and are probably caused by inadequate mixing of cells and the chemical under test. In any case, in the method proposed in these studies special care was taken to study microscopically the slides made from dilutions of these compounds which completely inhibited phagocytosis to be assured that at the toxic end point irreversible loss of phagocytosis through destruction of the cell had actually taken place.

The use of tests of the type described, which give accurate and reproducible results, offers a means of interpretation of the relative merits of a group of antiseptics under the conditions of use on or in the human body, since we are using a viable human tissue and estimating toxic effect on the cell upon which nature relies as one of her first lines of defense against invading microorganisms. It is apparent that the use of the method proposed in these studies will result in lower toxic end points than those obtained by the method previously described.[1] However, the former method, which is somewhat easier to perform, is of value in determining relative toxicity of a group of antiseptics designated for a particular purpose, while the method described in these studies may be taken as an indication of absolute toxicity, since actual destruction of a human cell is involved. The greater value in this method rests in its ability to approach more closely conditions of the use of an antiseptic on human tissue. When the actual resistance of human tissue to the action of an antiseptic is compared

with the resistance of pathogenic microorganisms to the same chemical, an evaluation of the efficacy of the antiseptic becomes possible.

SUMMARY

A method for determining the toxicity of antiseptics, which utilizes the destruction of leucocytes as an indication of toxic activity using human blood as a tissue, is presented.

Irreversible loss of the function of phagocytosis following a ten-minute contact with the compound tested is taken as an indication of death of the cell.

The toxicity of 26 antiseptics has been determined by the proposed method, and the results have been compared with those obtained by a method previously described.

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CHEMICAL

DETERMINATION OF CHOLESTEROL IN WHOLE BLOOD, SERUM, OR PLASMA*

H. GERSHBERG, M.D., AND J. C. FORBES, PH.D.
RICHMOND, VA.

DURING recent years many modifications of Bloor's procedure for the determination of blood cholesterol have been described. The majority of these do not differentiate ester from free cholesterol. Since the esterified form gives more color with the Liebermann-Burchard reagents, it is evident that the value for total cholesterol, as determined by these methods, must be too high. Other factors often leading to high results are colored substances which may be present originally in the blood, as in jaundice, or produced during the extraction process. Schoenheimer and Sperry,[1] and Kelsey,[2] avoid these difficulties by saponifying the esters and removing the cholesterol as the digitonide, which is then purified and analyzed. Ireland[3] first removes the interfering colors with silica, then saponifies the esters and extracts the cholesterol with chloroform. These procedures are quite long and do not lend themselves readily to the needs of the blood chemistry laboratory.

Some years ago one of us (J. C. F.) described a method[4] which eliminated interfering colors by use of a synthetic zeolite (Doucil). In this method esters are not saponified before color development, hence the results are necessarily high. In order to overcome this difficulty the method has been modified to obtain the cholesterol in a free state before solution in chloroform and color development. An added advantage is that free cholesterol can be determined on the same extract.

EXPERIMENTAL

Preparation of Extract.—One cubic centimeter of whole blood, serum, or plasma is added with stirring to approximately 17 c.c. of acetone –95 per cent alcohol (1:1) in a 25 c.c. volumetric flask. The extract is brought to a boil in hot water and set aside to cool. It is then adjusted to the 25 c.c. mark, mixed thoroughly, and filtered through a rapid filter paper. Ten cubic centimeters of the filtrate are measured into a 25 c.c. Erlenmeyer flask, and 0.2 c.c. of 15 per cent potassium hydroxide is added. The solution is warmed in hot water and placed in an incubator at approximately 55° C. for about one and one-half hours. The flask is then removed, and the alkali is neutralized with 0.4 c.c. of hydrochloric acid so adjusted that it will assure complete neutralization. The hydrochloric acid concentration is approximately 4.9 per cent. After neutraliza-

*From the Department of Biochemistry, Medical College of Virginia, Richmond.
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tion the flask is placed in water at about 65° to 70° C., and the contents are evaporated to dryness with the aid of a stream of air played upon the surface of the liquid. The flask is removed, allowed to cool, and 0.4 c.c. of distilled water is added. About 1.3 Gm. of neutralized doucil are added and mixed by moderate shaking. Ten cubic centimeters of chloroform are added, and the flask is stoppered immediately with a cork. The mixture is given a whirling motion off and on for five to ten minutes after which it is filtered through a rapid filter paper. In order to prevent concentration of the solution during filtration the funnel should be covered with a watch glass and filtration should be stopped by removal of the filter paper from the funnel as soon as the greater part of the solution has passed through. Five cubic centimeters of the filtrate are pipetted into a test tube standing in a wire rack in a water bath at 21° to 22° C. Five cubic centimeters of a standard cholesterol solution in chloroform (1 c.c. = 0.08 mg. of cholesterol) are pipetted into another test tube placed in the same bath. Two cubic centimeters of acetic anhydride are added to each. The tubes are mixed thoroughly and replaced in the bath for a time sufficient to enable the contents of the tubes to come to the temperature of the bath. This usually does not require more than five minutes, but no harm is done if they are left for a much longer period. Even a two-hour period does not affect the final color development. Four small drops of concentrated sulfuric acid, approximately 0.1 c.c., are added to each tube from a dropping bottle with a ground glass pipette. The tubes are then removed one at a time, the contents mixed thoroughly, and replaced in the bath. The bath is placed immediately into a dark cupboard for twenty minutes, after which the colors are compared in a colorimeter with an Eastman 71A filter in the eyepiece. The temperature of the bath at the time of comparison should not have risen above 23° C.

When free cholesterol is to be determined, 5 c.c. of the filtrate are measured into a 15 c.c. centrifuge tube, the free cholesterol precipitated as the digitonide and determined by the method of Schoenheimer and Sperry.[1] After solution of the cholesterol digitonide in acetic acid, it has been found advisable to control the temperature by using a water bath in the manner already described, except that a temperature of 25° C. is used and at least thirty minutes are allowed for color development.

Recovery of Cholesterol From Standard Solutions.—In order to show the complete extractability of cholesterol, aliquots of the standard were evaporated to dryness in 25 c.c. Erlenmeyer flasks, the residue was dissolved in 1:1 alcohol-acetone mixture and was carried through exactly the same procedures as those described for the unhydrolyzed and hydrolyzed extracts. Complete recovery was obtained in each case. The presence of water was found necessary for complete extraction. The amount of water used can be varied within fairly large limits; any amount between 0.4 and 1 c.c. inclusive allowed complete recovery of the cholesterol. Amounts outside these limits resulted in incomplete recovery. In the case of hydrolyzed extracts or alkali-treated standards, it was found necessary to neutralize the alkali before extracting with chloroform. We prefer to neutralize it before evaporation of the alcohol-acetone extract, but equally satisfactory results were obtained by neutralization immediately after evaporation.

TABLE I

CHOLESTEROL CONTENT OF UNHYDROLYZED BLOOD SERUM AS DETERMINED WITH AND WITHOUT DOUCIL

SERUM NO.	CHOLESTEROL PER 100 C.C. OF SERUM		SERUM NO.	CHOLESTEROL PER 100 C.C. OF SERUM	
	WITHOUT DOUCIL (MG.)	WITH DOUCIL (MG.)		WITHOUT DOUCIL (MG.)	WITH DOUCIL (MG.)
1	168	168	16	120	120
2	243	244	17	148	139
3	154	150	18	227	222
4	208	208	19	188	185
5	171	181	20	405	397
6	160	163	21	173	173
7	170	172	22	162	164
8	319	319	23	398	393
9	289	289	24	170	169
10	227	227	25	170	163
11	208	203	26	211	213
12	232	225	27	241	240
13	242	244	28	150	150
14	282	276	29	147	146
15	185	188	30	137	137

1 to 15 are normal persons.
16 to 30 are nonjaundiced hospital patients.

TABLE II

COMPARISON OF APPARENT CHOLESTEROL CONTENT AS CALCULATED FROM COLOR DEVELOPMENT BEFORE AND AFTER HYDROLYSIS

SERUM NO.	BEFORE HYDROLYSIS (MG. PER 100 C.C.)	AFTER HYDROLYSIS (MG. PER 100 C.C.)	RATIO B/A	PERCENTAGE FREE*
1	244	194	1.25	--
2	276	226	1.22	--
3	180	136	1.32	--
4	220	179	1.23	--
5	225	177	1.27	--
6	203	160	1.27	--
7	129	107	1.21	--
8	195	160	1.22	25
9	282	225	1.25	25
10	175	140	1.25	29
11	270	204	1.32	25
12	280	237	1.18	23
13	750	717	1.05	85
14	173	150	1.15	82
15	257	245	1.05	81
16	115	100	1.15	79
17	112	101	1.11	73
18	175	153	1.14	72
19	209	195	1.07	64
20	165	152	1.09	56
21	346	323	1.07	54
22	336	318	1.06	52
23	177	162	1.09	51
24	123	115	1.07	51

1 to 12 are normal sera.
13 to 24 are from jaundiced patients with liver disease.
*Free cholesterol was determined by Schoenheimer and Sperry's method and is expressed as percentage of the value obtained for total cholesterol after hydrolysis.

Recovery of Cholesterol From Unhydrolyzed Extracts.—In these experiments 10 c.c. of the alcohol-acetone extract were evaporated to dryness. The apparent cholesterol content was then determined in the manner already described, except that no potassium hydroxide or hydrochloric acid was used. The values obtained for different sera are given in Table I along with those obtained by simply evaporating an aliquot of the alcohol-acetone extract, dissolving the residue in chloroform and developing the color in the usual way. Since no sera containing an abnormal amount of pigment were used, the amount of pigment present was insufficient to interfere with color comparison even in the absence of doucil. It will be seen that the agreement between the two methods is excellent.

Recovery of Cholesterol From Hydrolyzed Extract.—That complete recovery of cholesterol was obtained when a standard was put through the procedure outlined for the hydrolyzed extract has already been mentioned. Evidence for the complete extractability from hydrolyzed serum extracts is shown in Table II. In these experiments comparisons have been made of the color developed by the extract before and after hydrolysis. In 12 normal subjects the ratios varied from 1.18 to 1.32, averaging 1.25. Also included in Table II are a number of typical values on pathologic sera from patients with liver damage in whom a marked increase in the percentage of free cholesterol was obtained. It will be seen that the ratio in all patients is definitely below normal. A ratio of 1 would indicate complete absence of ester cholesterol. Since a slight error in either of the determinations affects the ratio materially, variations in ratio similar to these recorded must be expected.

TABLE III

APPARENT CHOLESTEROL VALUE PER 100 C.C. OF JAUNDICED SERUM DETERMINED WITHOUT HYDROLYSIS, WITH AND WITHOUT DOUCIL

SERUM NO.	APPARENT CHOLESTEROL CONTENT		ICTERIC INDEX (UNITS)
	WITHOUT DOUCIL (MG.)	WITH DOUCIL (MG.)	
1	261	241	160
2	342	327	152
3	326	301	112
4	139	125	68
5	279	270	44
6	240	232	32
7	138	136	24
8	129	114	22
9	125	112	22

Removal of Interfering Substances by Doucil.—It is well known that certain colored substances often present in blood, such as bile pigments, or those produced during the extraction, especially when whole blood is used, will cause high results by interfering with colorimetric readings. This is especially true if a suitable filter is not used in the eyepiece. When doucil is used as described, these interfering substances do not dissolve in the chloroform, and consequently do not interfere with the accuracy of the results. Table III presents values obtained for unhydrolyzed extracts on a number of jaundiced sera with and without the use of doucil. It will be seen that the values for sera of high icteric index are distinctly higher in the absence of doucil. In Table IV are shown a

few typical results showing that doucil will remove the brownish color which forms when whole blood is boiled with alcohol-acetone solution. In these cases three samples of whole blood were taken and added to the alcohol-acetone mixture. One flask was brought just to a boil, another was boiled for five minutes, and the third was boiled for twenty minutes. After cooling, all were diluted to volume and mixed thoroughly. Each was then filtered, and the apparent total cholesterol content was determined without hydrolysis, with and without doucil. It will be seen that when doucil was used, the value obtained for cholesterol was not affected even by long boiling of the blood, but values obtained in the absence of doucil increased progressively as the time of boiling was lengthened, with production of greater amounts of colored products.

TABLE IV

APPARENT CHOLESTEROL VALUE OF WHOLE BLOOD EXTRACTS DETERMINED WITHOUT HYDROLYSIS, WITH AND WITHOUT DOUCIL

Values are expressed in milligrams per 100 c.c. of whole blood

BLOOD NO.	APPARENT CHOLESTEROL VALUE					
	A		B		C	
	WITHOUT DOUCIL	WITH DOUCIL	WITHOUT DOUCIL	WITH DOUCIL	WITHOUT DOUCIL	WITH DOUCIL
1	166	161	189	161	181	164
2	234	225	243	215	249	222
3	128	123	138	124	146	124

A, Brought just to a boil during the alcohol-acetone extraction.
B, Boiled for five minutes during the alcohol-acetone extraction.
C, Boiled for twenty minutes during the alcohol-acetone extraction.

SUMMARY

A relatively simple method for the determination of free and total cholesterol is described. Interfering substances, such as bile pigments or colored substances, produced during the extraction process are removed by means of a commercial synthetic zeolite (doucil).

Commercial doucil is alkaline after drying. Neutralized doucil suitable for use may be obtained from A. H. Taylor & Co., Baltimore, Md.

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THE USE OF A NEW DIGESTIVE MIXTURE FOR PROTEIN DETERMINATIONS*

JOSEPH BENOTTI, B.S., M.S., BOSTON, MASS.

THE best clinical nitrogen determinations, whether they be of a protein or nonprotein nature, are measured by acid digestion, and the resulting ammonia is determined by the Kjeldahl method or is nesslerized by direct addition of Nessler's solution.

Several digestion mixtures are available to the technician for nitrogen determinations, all of which have their advantages and disadvantages. The speed with which a certain sample of material will digest, that is, change from organic to inorganic material, depends upon the concentration of phosphoric acid present. Folin's digestive mixture[1] has a rather high concentration of phosphoric acid in comparison to the sulfuric acid present. This makes a very powerful and rapid digestive mixture. However, such a high concentration of phosphoric acid usually attacks glass and produces cloudiness in the resulting solution unless the digestion is stopped at the proper time. Dupray[2] has reduced the phosphoric acid content of his digestive mixture for nonprotein solutions so as not to produce the above effects. However, by so doing he has also reduced the speed of digestion itself. To overcome this he added an oxidizing agent, perchloric acid. This makes a good mild acid digestive mixture.

Since proteins have such a high nitrogen content, they require a digestive mixture which is very strong. However, it was my intention to make a strong digestive mixture which would not have too high a concentration of phosphoric acid that would attack pyrex glass, and still would digest with some speed. Furthermore, it must produce clear nesslerized solutions.

In all the various acid mixtures tried the phosphoric acid content was kept to a minimum so as to prevent the etching of glass due to overdigestion. Several oxidizing agents were tried to offset the deficiency of phosphoric acid. Some worked very nicely as far as the digestion was concerned, but on later nesslerization gave considerable cloudiness which rendered it impossible for use in this method since the nitrogen is finally measured by direct nesslerization. Finally, a very fine digestive mixture was realized which had the following composition: 54 c.c. of water, 35 c.c. of sulfuric acid concentrated, 10 c.c. of phosphoric acid 85 per cent, and 0.5 c.c. of perchloric acid 20 per cent. This digestive mixture does not attack glass, and on gentle boiling there is no danger of overdigestion. It gives absolutely clear solutions upon the addition of water after partial cooling. The use of digestive mixtures which have high concentrations of phos-

*From the Chemistry Laboratory of the Joseph H. Pratt Diagnostic Hospital, Boston.
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phoric acid force one to add water after digestion while still hot in order to avoid the formation of insoluble precipitates. It is thus possible to lose nitrogen due to spurting.

The digestive mixture herein described has one disadvantage and this is that following digestion and dilution with water the resulting solution must be ice-cooled before nesslerization since the nesslerized solutions sometimes turn cloudy when they are not previously cooled. The resulting digested solutions are nesslerized and read either on a colorimeter or photelometer. The nitrogen values obtained by this procedure were checked by the Kjeldahl method and compared very well.

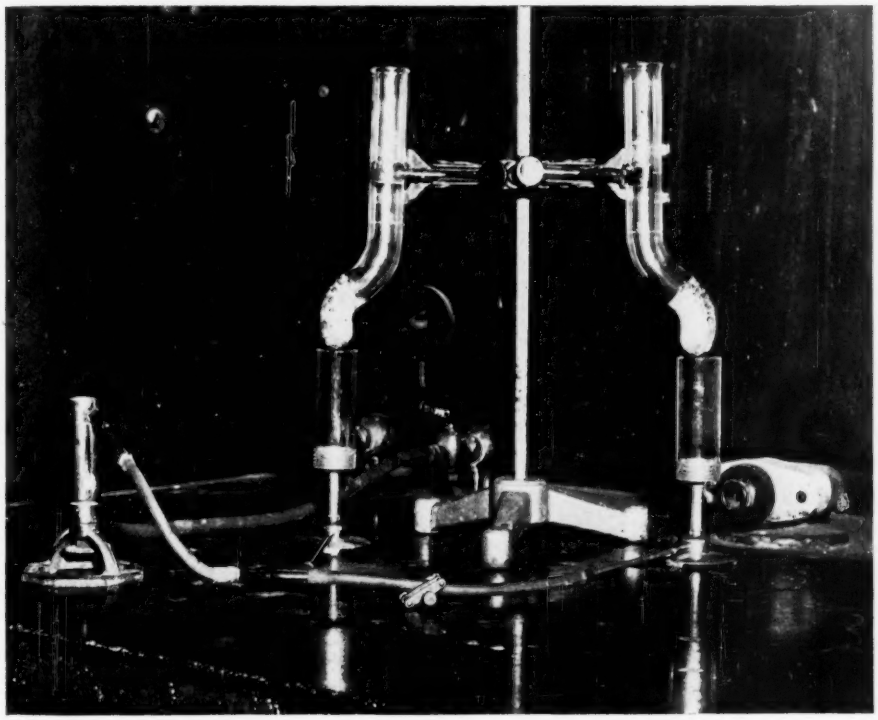

Fig. 1.—Apparatus used for protein digestions.

TOTAL PROTEIN

Dilute 1 c.c. serum to 50 c.c. with water and mix well. Take 1 c.c. of diluted serum in a special digestion tube,* previously described.[3] Add 1 c.c. of digestive mixture described above. Digest by boiling gently over a micro chimney burner, as shown in Fig. 1, and cover with a watch glass when charring begins. This takes about fifteen minutes. When it is moderately cool, dilute with distilled water to 35 c.c. Ice cool the latter solution and add 15 c.c. of Nessler's reagent. For comparison in a colorimeter one must use 2 c.c. of standard ammonium sulfate solution, which contains 0.1 mg. nitrogen per cubic centimeter plus 1 c.c. of acid digestive mixture; dilute to 35 c.c., ice cool, and nesslerize. The

*Special digestion tubes may be purchased from Howe and French, 99 Broad Street, Boston.

nitrogen may also be read in a photelometer using instead of the standard a blank which is made up of 1 c.c. of acid mixture; dilute to 35 c.c. with water, ice cool, and nesslerize. Calculation: $S/U \times 0.2 \times \frac{100}{0.02} \times 0.00625 - 0.2 =$ Gm. of total protein per 100 c.c. (0.2 represents the equivalent amount of protein due to the average nonprotein nitrogen).

ALBUMIN

One cubic centimeter of serum is added to 29 c.c. of 22.2 per cent sodium sulfate. Incubate at 37° C. for three or more hours. Filter and discard the first 10 c.c. of filtrate since it will be low in albumin due to absorption by the filter paper. Take 1 c.c. of clear filtrate, add 1 c.c. of digestive mixture described above, and digest in a special digestion tube[3] as described for total protein. Dilute to 35 c.c., ice cool, and nesslerize. The standard is the same as for total protein.

Calculation: $S/U \times 0.2 \times \frac{100}{0.033} \times 0.00625 - 0.2 =$ Gm. per 100 c.c. of albumin. Total protein minus albumin gives globulin.

SUMMARY

A new digestion mixture is herein described which digests protein and nonprotein solutions with the same ease. It does not attack glass, and the digestion does not have to be stopped at the proper moment in order to prevent cloudiness upon the addition of water. A method is described for total protein, albumin, and globulin which is easy, rather rapid, and accurate since there is no loss of material during digestion. This digestion mixture has recently been used for nonprotein nitrogen determinations with great success.

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QUALITATIVE STUDIES OF THE BILIRUBIN IN BODY FLUIDS

I. A Comparison of the Direct Diazo Reaction by the Photoelectric Colorimeter, the Three Test Tube Method, and the Oxidation Test in Serum

Georg Lepehne, M.D., Boston, Mass.

IN 1918 Hijmans van den Bergh[1] reported that the addition of diazo reagent to the blood serum of a patient with obstructive jaundice was followed by the prompt appearance of a characteristic color. When the same reagent was added to the serum of a patient with hemolytic jaundice, the characteristic color appeared very slowly, and then only after a considerable lapse of time. When 95 per cent alcohol was added to the serum of a patient with hemolytic jaundice along with the diazo reagent, the characteristic color developed promptly. On the basis of these observations van den Bergh classified the reaction as "direct" when no alcohol was needed to obtain a prompt appearance of color, and as "indirect" when alcohol was required. Since then the literature contains such expressions as "bilirubin direct positive," "direct negative," or "bilirubin indirect positive." These terms, when applied to the qualitative (direct) diazo reaction are confusing.[2] The logical terms are direct diazo reaction prompt or delayed. In addition to these two types of reaction, Feigl and Querner,[3] and others[4, 5] described the biphasic reaction, which is a combination of the prompt and delayed reaction.

Since the existing terminology is confusing and since the diagnostic value of the qualitative van den Bergh test is still a matter of dispute, the direct diazo reaction was studied by the photoelectric colorimeter and compared with the three test tube method.[7] The results reported herein will show the comparative value and usefulness of these two methods, and they will also indicate the advantages of using a uniform terminology. In addition, an oxidation test for further differentiation of the various types of bilirubin in blood serum will be described.

METHODS

Photoelectric Colorimeter.—The technique described by Malloy and Evelyn[6] was used. Readings were made at ten, twenty, and forty seconds, and at one, two, three, four, five, ten, and thirty minutes after the diazo reagent had been added to serum. It seemed advisable to obtain colorimetric determinations during the first minute of the reaction, since the qualitative difference between the prompt and delayed type is most striking during this interval. Since Malloy and Evelyn found that the prompt direct diazo reaction attained a maximum of color in thirty minutes, subsequent readings were not considered to be essential.

*From the Department of Medicine, Boston University School of Medicine.
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Three Test Tube Method.—This method originally described by the author[7] in 1923 makes use of the fact that caffeine salts (caffeine sodium benzoate or caffeine sodium salicylate) convert a delayed direct diazo reaction into one which is more or less prompt (Adler and Strauss[8]). Three small test tubes are used. One contains serum and diazo reagent; the second contains serum, diazo reagent, and caffeine salt; the third, serum diluted with water. Using the second and third tubes for purposes of comparison, the nature of the reaction in the first tube can be determined very easily.

TABLE I

COMPARISON OF QUALITATIVE AND QUANTITATIVE REACTIONS

CASES WITH PROMPT DIRECT DIAZO REACTION

DISEASE	TOTAL BILIRUBIN (MG. PER 100 C.C.)	1 MIN. DIRECT/30 MIN. INDIRECT (%)	1 MIN. DIRECT/ 1 MIN. INDIRECT (%)	1 MIN. DIRECT/30 MIN. DIRECT (%)	30 MIN. DIRECT/30 MIN. INDIRECT (%)
Catarrhal jaundice	11.8	52.3	76.6	72.4	72.2
Obstructive jaundice (cancer)	6.5	68.0	90.0	64.4	100.0
	15.7	58.3	83.0	70.1	81.2
	25.6	54.5	76.9	72.1	75.7
	18.1	46.6	69.7	60.2	77.5
	19.5	42.6	65.0	57.7	73.8
Obstructive jaundice (stone)	32.0	42.1	61.8	67.8	62.1
Cardiac jaundice	3.2	50.0	65.4	66.8	70.1
Cirrhosis of the liver with jaundice	7.8	46.9	65.9	66.6	70.4
Average		51.3	72.7	66.5	75.9

RESULTS

Prompt reactions were obtained in sera from 6 patients with obstructive jaundice caused by either cancer or stone, one patient with so-called catarrhal jaundice, one patient with liver cirrhosis with jaundice and ascites, and one patient with mild cardiac jaundice. The curves obtained by the photoelectric method showed a steep ascent, a high level in ten to twenty seconds (0.9 to 9 mg. per 100 c.c.), reaching a still higher level within one minute (1.5 to 14 mg. per 100 c.c.). Qualitatively, similar curves were obtained in the patients with obstructive, catarrhal, and cardiac jaundice, even though in the last case the total bilirubin content was only 3.2 mg. per 100 c.c. (Charts 1 and 2). These curves were almost parallel to the curves of the indirect (quantitative) diazo reaction, but, as a rule, they did not reach the same level. There always remained a small amount of bilirubin which reacted with the diazo reagent only after the addition of alcohol. The total bilirubin content of the blood in these cases ranged from 3.25 to 32 mg. per 100 c.c. (see Table I). In order to determine the significance of the various direct curves and their clinical value the figures, as obtained at the beginning of the reaction (one minute) and at the end (thirty minutes), were correlated with each other and with indirect (quantitative) photoelectric curves at one and thirty minutes. Table I shows that in the prompt direct curves, the one minute value reaches on the average 51.30 per cent of the total bilirubin content, 72.73 per cent of the indirect first minute value, and 66.51 per cent of the thirty minute direct readings. When the thirty minute values were

compared with each other, it appears that about 25 per cent of the total bilirubin is usually revealed by the indirect method only.

Further investigation must show whether differences can be determined between the prompt diazo reactions in hepatic and obstructive jaundice. Cantarow, Wirts, and Hollander[9] were unable to find any differences, but Varela and his co-workers,[10] and recently Heilbrun and Hubbard,[11] demonstrated that differences between these types of jaundice exist when they separated the "prompt" and the "delayed" bilirubin quantitatively by the method of chloroform extraction.

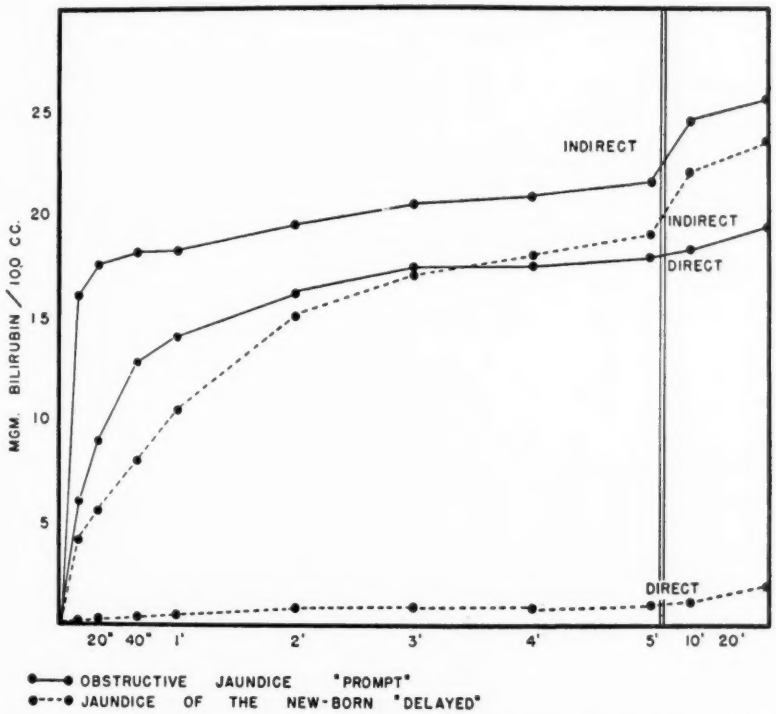

Chart. 1.—Comparison of the "prompt direct diazo reaction" in a case of obstructive jaundice with the "delayed direct diazo reaction" in a case of jaundice of the newborn.

In using the three test tube method, a prompt direct diazo reaction was characterized by the appearance of a color immediately after the addition of the diazo reagent. One has the impression that the redness reaches a maximum in one minute (van den Bergh and many other authors). The photoelectric curves, however, reveal that the maximum is reached in about thirty minutes, and Table I shows that the one-minute value of the direct curves is on the average only 66.5 per cent of the thirty-minute direct readings. This fact is due to the accuracy of the photoelectric colorimeter method for detection of the depth of color.

In the three test tube method a new observation was made which may be helpful in the classification of the different types of the direct diazo reaction. In the prompt direct diazo reaction the first orange-red color of the test tube containing the caffeine salts changes to purple in one to two seconds, while in

the tubes containing only serum and reagent the change appears in thirty to ninety seconds. In the delayed and biphasic reactions the appearance of the purple color is much more delayed.

To the *group of delayed direct diazo reaction* belong the serum of a patient with familial nonhemolytic jaundice, 4 patients with jaundice of the newborn, and one patient with healing hepatic jaundice. Furthermore, the following cases of latent jaundice belong in this group: 4 cases of pernicious anemia, 3 specimens of blood from the umbilical cord (placenta blood), one sulfanilamide jaundice (mild case, jaundice of hemolytic origin), one case of possible hemolytic anemia before and after splenectomy. The photoelectric direct curves show a striking contrast to the curves of the prompt group (see Charts 1 and 2). Not only in the first minute but also in the first five minutes a very slow ascent of the curves takes place, and after thirty minutes a low peak is reached. No difference was observed between the curves in the jaundiced cases and those with latent jaundice. But in the cases of jaundice of the newborn and in the case of healing hepatic jaundice the one minute values and the thirty minute values were relatively higher than in the other cases. Apparently the higher total bilirubin content in the jaundice of the newborn and in the healing hepatic jaundice causes the higher readings of the direct curves of these cases.

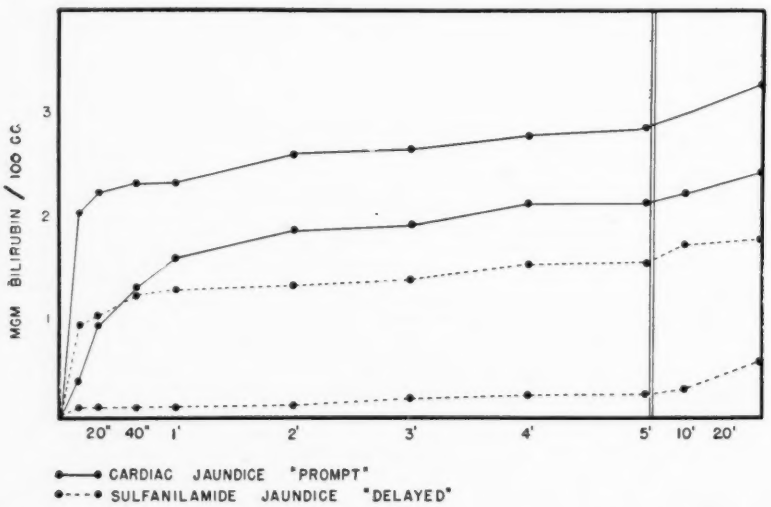

Chart 2.—Comparison of the "prompt direct diazo reaction" in a case of cardiac jaundice with the "delayed direct diazo reaction" in a case of sulfanilamide jaundice.

When the values obtained at various time intervals are correlated with each other, as was done in the cases of the prompt reaction, some striking results were obtained (Table II). In the delayed cases the value at one minute reaches only 9.5 per cent of the total bilirubin in contrast to 51.3 per cent in the prompt group. The other correlations show similar marked differences between the prompt and the delayed direct reaction. The thirty minute direct value reaches only 30.05 per cent of the total bilirubin (75.9 per cent in the prompt cases).

The three test tube method (direct diazo reaction) also shows that the redness in the delayed group develops slowly. The first trace of the orange-red coloring appears five to ninety seconds after the addition of the diazo reagent,

with an average of thirty-nine seconds. Then the color deepens slowly within one to twenty minutes (average five minutes) to a distinct orange red-color. It is of clinical interest that in this group the change to purple color occurs in about ten minutes to two and one-half hours, on the average in about one hour.

In the cases of jaundice of the newborn the development of the color after the addition of the diazo reagent is slower than in the other delayed cases. A distinct orange-red color could be noted only after ten minutes in Case 1 with 6 mg. total bilirubin; after twenty minutes in Case 2 with 7.25 mg.; after twenty-five minutes in Case 3 with 10.7 mg.; and after three hours in Case 4 with a total bilirubin of 23.36 mg. per 100 c.c. In the test tube method the speed with which the redness develops in jaundice of the newborn is apparently in inverse proportion to the total bilirubin content present. The change to purple color, which appears in the tube containing caffeine, does not occur at all in the other tube. Even twenty-four hours, thirty-six hours, and forty-eight hours after the addition of the diazo reagent, a dark orange-red or brownish-red color was present but no purple color. When a few grains of caffeine sodium benzoate were added to an orange-red twenty-four-hour specimen, a change to purple occurred two hours later.

TABLE II

COMPARISON OF QUALITATIVE AND QUANTITATIVE REACTIONS

CASES WITH DELAYED DIRECT DIAZO REACTION

DISEASE	TOTAL BILIRUBIN (MG. PER 100 C.C.)	1 MIN. DIRECT/30 MIN. INDIRECT (%)	1 MIN. DIRECT/1 MIN. INDIRECT (%)	1 MIN. DIRECT/30 MIN. DIRECT (%)	30 MIN. DIRECT/30 MIN. INDIRECT (%)
Pernicious anemia	1.3	8.2	10.0	16.2	51.4
	1.3	21.9	33.7	41.9	52.2
	2.1	5.2	8.0	18.5	28.2
	2.4	9.3	18.5	37.7	24.6
Hemolytic anemia	1.5	6.4	8.6	18.1	36.1
Hemolytic anemia after splenectomy	1.6	16.5	18.3	38.8	42.5
Sulfanilamide jaundice	1.8	7.2	10.5	23.7	30.3
Placental blood	0.9	18.4	26.4	41.9	46.2
	1.9	8.4	9.0	24.1	35.0
	2.2	11.4	13.6	52.3	22.8
Healing hepatic jaundice	5.2	11.3	16.7	43.7	26.0
Jaundice of newborn	7.5	10.4	16.0	56.1	18.5
	6.0	4.3	3.1	13.2	33.0
	10.7	7.9	12.7	38.8	18.0
	23.3	2.5	5.5	37.0	6.8
Average		9.5	13.4	33.4	30.5

This very delayed direct diazo test tube reaction in the cases of jaundice of the newborn seems to be in a certain contrast with the development of color obtained by the direct photoelectric method (average 0.618 mg. per 100 c.c. in one minute, and 1.5 mg. per 100 c.c. in thirty minutes). To explain this discrepancy it may be considered that the deep yellow color of these jaundiced sera may disguise the development of the red coloring in the test tube method.

Biphasic reactions were obtained in the sera from the following cases: one of liver cirrhosis, 2 of cholelithiasis with decreasing jaundice, and one of jaundice caused by metastases in the liver. In these cases the direct curves

ranged between the steep ascending curves of the prompt group and the low curves of the delayed group (see Chart 3 and Table III).

In this group the three test tube methods showed two variations which I described in 1921:[5] "biphasic prompt" and "biphasic delayed." In three cases (cholelithiasis with jaundice, liver metastases with jaundice) the reaction was biphasic prompt; immediately after the addition of the diazo reagent a slight red color appeared. The redness deepened more slowly than in the prompt cases but more rapidly than in the delayed cases. The maximum amount of color seemed to be reached after one to two minutes. The change to purple color

TABLE III

COMPARISON OF QUALITATIVE AND QUANTITATIVE REACTIONS

CASES WITH BIPHASIC DIRECT DIAZO REACTION

DISEASE	TOTAL BILIRUBIN (MG. PER 100 C.C.)	1 MIN. DIRECT/30 MIN. INDIRECT (%)	1 MIN. DIRECT/1 MIN. INDIRECT (%)	1 MIN. DIRECT/30 MIN. DIRECT (%)	30 MIN. DIRECT/30 MIN. INDIRECT (%)
Cholelithiasis with jaundice	6.7	15.9	19.4	59.7	26.7
Jaundice caused by cancer metastases	6.1	28.8	35.4	65.9	43.7
Cirrhosis of the liver	2.2	34.0	47.9	60.4	55.4
Cholelithiasis	1.1	35.7	56.8	58.0	61.6
Average		22.9	39.9	62.5	46.8

appeared in about one and one-half to four minutes, that is, a little later than in the prompt group. The total bilirubin content in these cases was high (see Table III). In the other cases (2 of liver cirrhosis, 2 of cholelithiasis) the biphasic-delayed direct diazo reaction was present: immediately after the addition of the reagent a very slight red color appeared, but the further development of the color was very slow, similar to the delayed reaction. The maximum color seemed to be reached in two minutes or later. The change to purple in 2 cases with a total bilirubin content of 1.1 mg. and 2.2 mg. per 100 c.c. required twenty and forty-five minutes, respectively.

Besides the differences which can be shown with the direct diazo reaction, van den Bergh[1] reported that the "prompt" bilirubin can be rapidly oxidized to biliverdin, while the "delayed" bilirubin is oxidized slowly. In 1914 Beneke[12] reported that the bilirubin present in blood of jaundice of the newborn, after being treated with formalin, remained yellow, while the bilirubin of cases with obstructive jaundice became green. In 1922 I[5] proposed an easy oxidation test, and as a result of accumulated experience with this test found that it is of value in differentiating the various forms of bilirubin.

METHOD

The reagent is made by adding 1 c.c. of 0.5 per cent sodium nitrite solution to 9 c.c. of 1 per cent nitric acid solution. The reagent is prepared freshly each time it is used. The test is performed as follows: 0.2 to 0.3 c.c. of the serum is placed in each of two small test tubes. One tube is diluted with the same amount of water. To the other tube is added 0.2 to 0.3 c.c. of the reagent. Both tubes are held in front of a white paper against the daylight, and the rapidity with which a green color develops is noted during a five to ten minute period.

RESULTS

In a series of 80 examinations 3 different reactions were observed: (1) "Prompt oxidation test": immediately after the addition of the reagent, a definite green color develops and reaches its maximum intensity in ten to fifteen seconds. The depth of the color depends on the amount of bilirubin present, but it is always green. (2) "Diminished-delayed oxidation test": either a very slight yellow-greenish color develops immediately and changes to a grayish color within two to three minutes, or a rather slight yellow-greenish color develops in thirty seconds to fifteen minutes after the addition of the reagent. (3) "Negative oxidation test": after the addition of the reagent the color of the serum does not change at all or only becomes lighter. Within the next one to three minutes the color changes gradually to a grayish or straw color. No green color is present at any time. Addition of alcohol or of caffeine salts has no influence on the diminished-delayed or on the negative oxidation test.

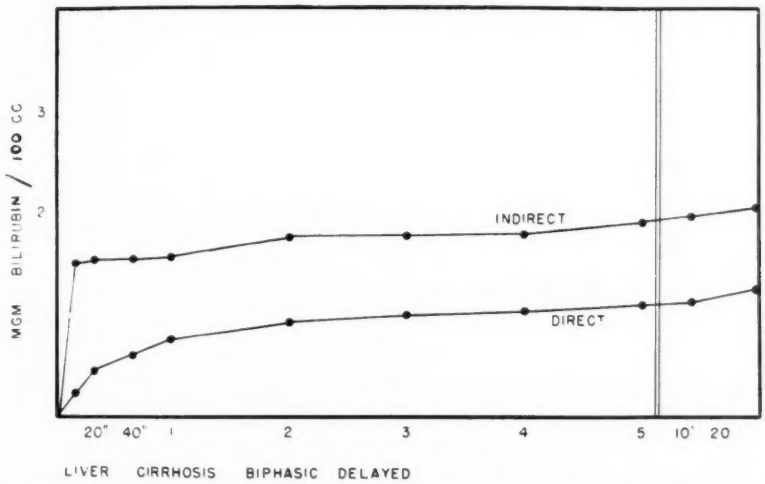

Chart 3.—"Biphasic-delayed direct diazo reaction" in a case of liver cirrhosis.

The *prompt oxidation test* occurred in all cases of obstructive and hepatic jaundice and corresponded to the prompt direct diazo reaction. In one case of mild cardiac jaundice with the low total bilirubin content (3.25 mg.) and in 2 cases with a biphasic-prompt direct diazo reaction (cholelithiasis with 6.7 mg. and cancer metastases in the liver with 6.10 mg. per 100 c.c.) a prompt oxidation test was present. In serum of obstructive jaundice diluted to a bilirubin content of less than 2 mg. per 100 c.c., the prompt oxidation test changed in the majority of the cases to a diminished-delayed reaction.

The *diminished-delayed oxidation test* was observed in cases with delayed or with biphasic-delayed direct diazo reaction (2 cases of liver cirrhosis with total bilirubin of 1.5 mg. and 2.3 mg.; one case of pernicious anemia with a total bilirubin of 1.36 mg.; one case of nonhemolytic familial jaundice with a total bilirubin of 4.06 mg.; one case of sulfanilamide jaundice with a total bilirubin of 1.82 mg.; and one case of healing hepatic jaundice with 5.22 mg. bilirubin per 100 c.c.). The time between the addition of the reagent and the

appearance of the yellow-greenish color did not depend on the amount of the total bilirubin. No case with a prompt or biphasic-prompt direct diazo reaction showed an oxidation test of this type.

The *negative oxidation test* was present in cases with delayed direct diazo-reaction only: pernicious anemia, hemolytic anemia before and after splenectomy, liver cirrhosis, in all specimens of cord blood of the newborn and in the cases of icterus neonatorum. The high total bilirubin content in the cases of jaundice of the newborn (6 mg. to 23.3 mg. per 100 c.c.) shows that also the negative oxidation test was independent of the bilirubin content of the serum. In one exceptional case of cholelithiasis with a total bilirubin content of 1.1 mg. a negative oxidation test was found in the presence of a biphasic-delayed direct diazo reaction.

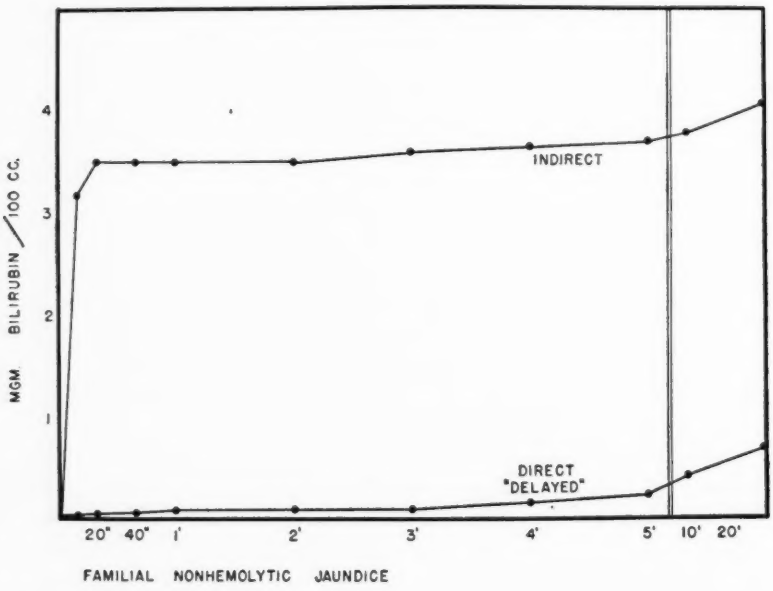

Chart 4.—"Delayed direct diazo reaction" in a case of familial nonhemolytic jaundice.

Photoelectric curves of the different types of this oxidation reaction could not be obtained even though different filters (620, 630, 635) were used. Some examinations with Fouchet's reagent (trichloracetic acid) and with the oxidation reagent of Malloy and Evelyn[13] (hydrogenium peroxide, concentrated hydrochloric acid, and alcohol) did not show consistent results. Andrewes[14] states that tincture of iodine gives a green color when added to a serum of obstructive jaundice, but an uncertain color when added to a serum of hemolytic jaundice. He obtained similar results with Fouchet's reagent.

DISCUSSION

The results presented in this paper show that a real negative direct diazo reaction does not exist. In all cases a positive direct diazo reaction can be obtained by the photoelectric method as well as by the test tube method, but the time necessary for the development of the color and its intensity vary markedly

in the different types of sera. When alcohol and diazo reagent are added to the serum ("indirect" or quantitative reaction), the color appears promptly in every serum. Therefore, it is fair to say that, as far as the qualitative test is concerned, it is not absolutely accurate to differentiate between "direct bilirubin" and "indirect bilirubin" or to speak of "bilirubin direct negative" or "bilirubin indirect positive." It is also confusing that Malloy and Evelyn[6] used the word "promptness" in describing the direct photoelectric curves in cases of hemolytic jaundice which give a definite delayed direct diazo reaction. Bilirubin in the blood serum, when tested by the direct method, is either "prompt," "delayed," or "biphasic."

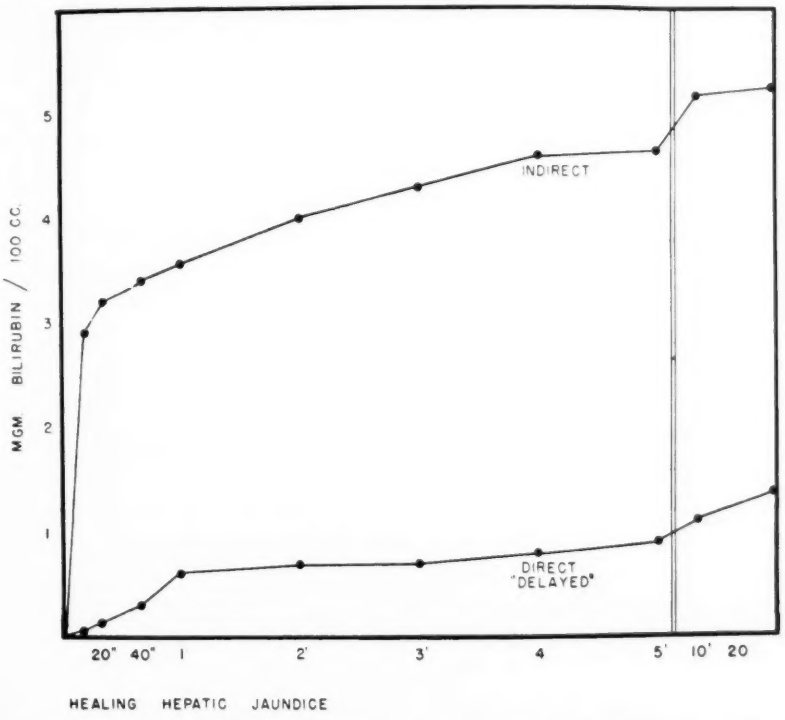

Chart 5.—Delayed direct diazo reaction" in a case of healing hepatic jaundice.

The qualitative van den Bergh test is believed to be of diagnostic value. When the incline of the photoelectric curves is studied, definite differences appear between the direct prompt, direct delayed, and direct biphasic reaction. By correlating the values at one minute with those obtained after thirty minutes, the difference between the three types of curves is striking. By means of the three test tube method, the prompt, the biphasic prompt, the biphasic delayed, and the delayed types can be distinguished, especially when one takes into consideration the time at which the orange-red color changes to purple. In addition to the direct diazo reaction the oxidation test may be of aid in classifying the different forms of jaundice.

The presence of a prompt or biphasic-prompt direct diazo reaction and a prompt oxidation test exclude an hemolytic jaundice or a jaundice caused by decreased functional ability of the liver cells to excrete bilirubin to the normal

amount (e.g., familial nonhemolytic jaundice) (see Chart 4). These types of the direct diazo reaction and of the oxidation test indicate liver cell damage or an obstruction of the bile ducts. This fact is of particular clinical importance in cases of latent jaundice with a relatively low total bilirubin content. In such cases as cholelithiasis, cardiac failure, liver metastases, and arsphenamine intoxication the direct test tube diazo reaction, if prompt or biphasic prompt, and the oxidation test can reveal the presence of liver cell damage at once. On the other hand, a definite delayed direct diazo reaction and a negative oxidation test (more rarely a diminished-delayed oxidation test) demonstrate the presence of a hemolytic jaundice or jaundice due to functional inability of the liver cells to excrete bilirubin in normal amount or a combination of both. The delayed (sometimes biphasic delayed) diazo reaction and the negative oxidation test exclude hepatic or obstructive jaundice. Only in the early stages and in the healing stages of hepatic or obstructive jaundice a biphasic delayed or even a delayed direct diazo reaction and a diminished-delayed or even negative oxidation test can occur (see Chart 5). In such cases the clinical picture and repeated qualitative examinations of the blood bilirubin in combination with quantitative examinations (icterus index) may help in arriving at the correct diagnosis.

SUMMARY

A study of the direct diazo reaction was made with the photoelectric colorimetric method, the three test tube method, and a qualitative oxidation test. The following facts emerged:

1. A prompt, a delayed, and a biphasic type of the direct diazo reaction can be distinguished.

2. The oxidation test can be prompt, negative, or diminished-delayed in various types of jaundice.

3. These methods are of diagnostic value, the most important being the photoelectric colorimetric method.

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STUDIES ON THE CLINICAL SIGNIFICANCE OF THE SERUM PROTEINS*

I. The Protein Content of Normal Human Venous and Capillary Serum and Factors Affecting the Accuracy of Its Determination

Benjamin M. Kagan, M.D., Richmond, Va.

CHEMICAL changes in the blood are becoming increasingly important in our interpretation and treatment of disease. In order for us either to interpret our clinical data in terms of physiologic or chemical changes or to interpret laboratory data in terms of clinical significance, it is necessary that we study thoroughly the changes of each chemical substance in the body under all circumstances.

To do this we must first of all have laboratory techniques available which are accurate and particularly simple and rapid enough to allow extensive investigation and general use. The methods for hemoglobin, sugar, urine albumin, etc., are simple, rapid, and sufficiently accurate for clinical purposes. They are, therefore, widely done, and their significance is generally appreciated.

In the case of blood protein concentration there has until recently been no test which met all these requirements, and largely for this reason the importance of the blood proteins has been widely underestimated.

It is proposed in this report and in subsequent reports to show the importance of the blood proteins in disease. Herein we shall discuss the normal concentration of blood protein and its two constituents, albumin and globulin, the methods available for determining these concentrations, and details regarding the collection, preparation, and handling of the specimen so as to insure maximum accuracy and greatest ease in obtaining the result.

METHODS

The most accurate method for determining the concentration of total protein and its constituents, albumin and globulin, is the Kjeldahl. Unfortunately,

*From the Johns Hopkins and Sinai Hospitals, Baltimore.
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it is relatively difficult, expensive, and time-consuming. It is, therefore, not generally available nor useful in the sense that the hemoglobin and urinalysis tests are.

There are in existence several colorimetric methods which, while less difficult to use, do not approach the simplicity of usual routine tests, some of which are subject to errors up to 10 per cent.

In 1937 I described a falling-drop technique,[1, 2] which is probably the most rapid and simple method for determining the total serum protein concentration.[3] Duplicate samples of the same specimen analyzed by this method check well within 0.1 Gm. per 100 c.c., and the mean deviation of 107 different specimens of serum when determined by this method and by the macro-Kjeldahl was ±0.16 Gm. per 100 c.c. This determination can be done with as little as 0.1 c.c. of blood obtained from finger tip or vein and compares in simplicity with the hemoglobinometer.

It is regrettable that there is no simple test for determining accurately the absolute concentration of albumin alone.* There are, however, several simple tests which indicate whether or not the globulin concentration is above normal. To mention only two: the globulin precipitation test† and the formol gel test.‡ If by means of one of these tests it is determined that the globulin concentration is not elevated, then changes in the total protein concentration may be safely attributed to change in the albumin concentration. This will be discussed in detail in subsequent papers on the behavior of albumin and globulin in disease. First, however, let us consider the effect of the manner of collecting, preparing, and handling the specimen on the accuracy of any blood protein determination by any method.

DETAILS REGARDING COLLECTION, PREPARATION, AND HANDLING OF SPECIMEN

Rowe[7] and others have found that the protein content of a specimen is increased almost 0.2 Gm. per 100 c.c. for each minute that venous stasis is maintained by a cuff around the arm. This is due to loss of water from the vascular bed. It is, therefore, advisable that blood should be collected within one minute of the application of the tourniquet.

Peters and Van Slyke[8] have reviewed the physiologic factors which may affect the normal concentration of serum proteins. Hyperventilation (when sufficient to produce tetany), vigorous muscular exercise, and stasis of the blood caused by chilling are all capable of increasing the protein concentration. When the body temperature is raised above normal, a lowering of the protein concentration occurs; and when sweating causes sufficient loss of fluid, the opposite obtains. In cases where they are present, these factors need to be kept in mind.

When blood proteins are determined by any of the present colorimetric methods, it is necessary to use blood collected after a twelve-hour fast because

*The falling-drop principle has been applied for the determination of albumin and globulin, but the necessary precautions are too rigorous for routine use. Since only 27 determinations have been correlated with the Kjeldahl, this method cannot at this time be generally recommended.[4]

†Globulin precipitation test (Naumann[5]): Add 0.1 c.c. of serum to 1.0 c.c. of distilled water in a 10 mm. wide test tube. Saturate with alveolar air and read after fifteen minutes. If definite cloudiness appears, globulin concentration is said to be more than 3.0 Gm. per 100 c.c.

‡Formol-gel (Bing[6]): Add 2 drops of 40 per cent formalin to 1 ml. of serum and read after three hours' standing at room temperature. Globulin concentration is elevated if tube can be inverted without losing contents.

the change in amino acids in blood following food intake affects this method. Such changes in the amino acids do not affect the Kjeldahl or drop methods. In four patients on regular hospital diets, from whom blood was drawn every three hours during the day and hourly after the noonday meal for four hours, there was no change in the serum proteins greater than 0.2 Gm. per 100 c.c. when the determinations were done by the drop method.

The following two experiments done by the falling-drop method suggest that it is advisable that no amount of fluid greater than 500 c.c. be ingested at any one time within four hours of the sampling: (1) Each of 3 persons was given between 600 c.c. and 750 c.c. of water, and their blood proteins were followed at half-hour intervals. All 3 showed decreases in protein concentration which averaged for the 3 a value of 0.15 Gm. per 100 c.c. (2) Each of 3 persons with minor surgical ailments was given 1,500 c.c. of tap water by mouth within five minutes. Their blood changes were similar. The serum protein level fell gradually over the first hour after ingestion of the water to an average of 0.5 Gm. per 100 c.c. less than its original value and returned to the previous level two hours after ingestion. On a subsequent day the same persons were given 1,500 c.c. of normal saline solution in similar manner. The serum proteins fell gradually over a two-hour period to an average of 0.9 Gm. per 100 c.c. less than the original value and were still in 2 cases 0.1 Gm. per 100 c.c. below the original value four hours after the ingestion. Following the ingestion of 500 c.c. of tap water or normal saline, changes in protein concentration are definitely within the experimental error. In view of the foregoing experiments, therefore, it is recommended that no amounts greater than this be ingested within four hours of sampling.

TABLE I

COMPARISON OF THE PROTEIN CONTENT OF SERUM WITH HEPARINIZED AND OXALATED PLASMA

SUBJECT	PATIENT 1	PATIENT 2	PATIENT 3
METHOD	KJELDAHL	KJELDAHL	KJELDAHL
Serum	4.66	6.11	7.21
Oxalated plasma	4.63	6.06	7.12
Heparinized plasma	4.82	6.71	7.56

In many previous investigations and in a number of modern texts no distinction is made between protein values for plasma or serum. The following experiment shows that such a distinction is necessary. Blood was drawn from 3 patients. From blood drawn into a single syringe, serum, oxalated plasma, and heparinized plasma were prepared. The proteins were determined by the macro-Kjeldahl method. The results are shown in Table I. Oxalated plasma may contain the same or lower concentration of protein than serum, even though it would logically be expected to contain about 0.3 Gm. more per 100 c.c. because it contains fibrinogen. Furthermore, the values obtained for oxalated plasma are not as reliable as those for serum, probably because the amount of water withdrawn from the red blood cells varies with the amount of oxalate used as well as with the varying effect that oxalate has on the cells of different persons. The values obtained for oxalated plasma would correspond to those obtained for serum only if the same amount of blood were always mixed together with the same amount of oxalate with an accuracy not usual in the laboratory, and also if it were

true that the cells of every person responded in exactly the same manner to changes in surrounding osmotic pressure. Heparinized plasma may contain as much as 0.65 Gm. per 100 c.c. more protein than oxalated plasma because of a combination of these reasons. In a previous report[2] we found that values obtained for serum protein were definitely more consistent than those for plasma. For these reasons, serum should be preferred to oxalated plasma for use in determining the blood protein concentration.

A series of experiments were done to determine the effect of the age of the specimen on serum protein concentration. These experiments were done by the falling-drop method. A few specimens kept in rubber-stoppered tubes and in an icebox showed increases of over 0.2 Gm. per 100 c.c. when more than twenty-four hours old. Tubes containing the same bloods, but sealed with wax and kept in the icebox, showed no change even after three days. Specimens which were kept in rubber-stoppered tubes at room temperature for twenty-four hours occasionally showed changes as great as 0.7 Gm. per 100 c.c. When cotton-stoppered tubes, such as used in some hospitals, were left at room temperature, increases as high as 1.6 Gm. per 100 c.c. were noted in twenty-four hours. In view of these findings, it would appear safest to do the determinations within twenty-four hours and to keep the specimen in a well-stoppered tube in the icebox.

When a tube of blood is centrifuged without a cork, the concentration of serum protein may rise as much as 0.5 Gm. per 100 c.c. when spun at 3,000 r.p.m. for only fifteen minutes. Although a more detailed analysis of this factor was not attempted, the changes seemed to be directly proportional to the amount of exposed surface relative to the total volume of serum in the tube. When well corked, a tube of blood may be spun at this speed for half an hour without change in concentration.

When the blood was properly stored, there was no difference in the serum protein concentration whether the blood was centrifuged four or twelve hours after its collection or whether or not the serum was separated from the clot before it was stored.

Hemolysis, brought about by shaking a tube of blood vigorously, produced in general an increase of the protein concentration—occasionally of 0.5 Gm. per 100 c.c. Occasionally hemolysis resulted in slight lowering of protein concentration. Apparently the effect depends on the proportion of water and hemoglobin lost from the red blood cells in the procedure. Since it is impossible to judge how great an effect hemolysis will have on an individual specimen, it is considered safer never to use a hemolyzed specimen for this analysis.

In some cases where venous blood is difficult to obtain, it would be very useful to be able to determine the serum protein of blood obtained from the finger tip or heel. This is especially true in pediatrics. Since the amount of blood so obtainable is not adequate for the macro-Kjeldahl technique nor for sufficient accuracy by the colorimetric methods, no comparison of the serum protein concentration of venous and capillary blood has been heretofore reported.

With 0.2 c.c. of blood, which is easily obtainable from the finger tip, about three to five determinations of serum protein can be done by the drop method. It was found most convenient to collect this blood in small test tubes which

measure 35 mm. by 6 mm. and have round bottoms and no lip. A rubber cap fits over the mouth to prevent evaporation. If the finger be placed in warm water for a few minutes prior to being stuck, a free flow is obtained without squeezing tissue fluid into the wound. Capillary blood collected in this manner was compared with venous blood collected from the same arm at the same time. The venous blood was collected without the use of a tourniquet. In tests on 14 adults and 2 children the difference in serum protein concentration between venous and capillary blood was never more than 0.2 Gm. per 100 c.c., and the average difference was 0.07 Gm. per 100 c.c. It is concluded from this experiment that capillary blood is a satisfactory substitute for venous blood and for clinical purposes the results may be considered identical.

NORMAL VALUES OF TOTAL SERUM PROTEIN AND OF ALBUMIN AND GLOBULIN

In 1933 Peters and Eisenman[9] reported the value for serum proteins on 52 normal persons. They found that 90 per cent of the values were between 6.3 Gm. and 7.7 Gm. per 100 c.c. This is the largest series reported to date which is based on persons who were known to be normal. Larger series have been reported but not on normal subjects. The purpose of the present report is to add to the data already collected with determinations done by the falling-drop method on 150 ambulatory persons who were normal by examination.

Our analysis was done on the blood of 75 medical students, 25 student nurses, and 50 blood donors. On each student a physical examination, Wassermann test, and urinalysis were done. One student had had a recent appendectomy, one had inactive rheumatic heart disease, and two had orthostatic albuminuria. Their ages ranged from 17 to 35 years. Their physical status was considered good in 52 per cent, robust in 26 per cent, fair in 20 per cent, and asthenic* in 2 per cent.

The donors were not examined. They had never given blood before and were selected for the most part from men accustomed to hard manual labor in steel and tin plate mills. The results on their blood differ in no respect from that of the others.

The range of serum protein concentration in these 150 persons was 6.1 Gm. to 7.6 Gm. per 100 c.c. The average value was 6.7 Gm. per 100 c.c. The individual male whose serum protein level was 6.1 Gm. per 100 c.c. had had a severe, continuous nosebleed over the previous three days, which finally required cauterization. One month later his serum protein level was 7.0 Gm. per 100 c.c. The female whose protein level was 7.6 Gm. per 100 c.c. was in good health except for being 20 pounds under average weight for her age and height. Another analysis one month later gave a value of 7.5 Gm. per 100 c.c.

Bruckman and associates[10] report an average value of 6.93 Gm. per 100 c.c. for 13 males and 7.6 Gm. per 100 c.c. for 8 females. Peters and Eisenman found no sex difference in their series. The range for the 36 females in this series was 6.3 Gm. to 7.6 Gm. per 100 c.c., with an average of 6.7 Gm. per 100 c.c. The range for the 114 males was 6.1 Gm. to 7.5 Gm. per 100 c.c., with an average of 6.7 Gm. per 100 c.c.

*For the medical data on these students I am indebted to Dr. Warde B. Allan and Dr. N. B. Herman.

It will be noted that most of the possible deviations from the standard technique used here cause an increase in the serum protein concentration. This may explain why previous investigators have had a few more values between 7.5 Gm. and 8.0 Gm. per 100 c.c. than are found in this report. Many investigations reporting higher values than those reported by Peters and Eisenman and by us have been done on persons who were not normal but who had diseases of various sorts which were assumed at the time not to affect the serum protein concentration. That values above 7.5 Gm. per 100 c.c. are unusual in normal persons is further confirmed by the fact that of 995 determinations of total plasma and serum protein by the macro-Kjeldahl method on 576 patients at the Johns Hopkins Hospital only 24 determinations on 20 patients gave values between 7.5 Gm. and 8.0 Gm. per 100 c.c. Of these 20 patients 15 had diseases which would be expected to cause an increase in the serum protein concentration. This will be discussed in more detail in a subsequent report.

Throughout our work we have accepted the values for normal concentration of serum albumin and globulin to be those given by Peters and Eisenman. They were based upon 50 Kjeldahl determinations on specimens from 34 normal persons. They are for albumin 4.0 Gm. to 5.5 Gm. per 100 c.c. and for globulin 1.4 Gm. to 3.0 Gm. per 100 c.c.

SUMMARY

In order to minimize inaccuracies in the determination of blood protein which may arise during collection and handling of the specimen, it is advisable to proceed as follows:

1. Blood should be collected within one minute after the application of the tourniquet.

2. Serum gives more accurate results than oxalated plasma and should, therefore, be preferred.

3. Only nonhemolyzed serum should be used.

4. The blood should be kept in a well-stoppered tube in an icebox, and the analysis should be done within twenty-four hours after collection of the blood.

5. The tube containing the blood sample must also be well stoppered during centrifugation.

6. It is advisable that no amount of fluid greater than 500 c.c. be ingested at any one time within four hours of the sampling. When all the conditions stated are maintained, a difference of protein content of 0.2 Gm. per 100 c.c. from sample to sample in following the course of a disease may be considered quantitatively significant when either the falling drop or the Kjeldahl method is used for all the determinations. This, of course, does not apply when one determination is done by one method and another determination by another method.

Serum from capillary blood obtained from the finger contains for clinical purposes the same amount of protein as venous blood; and the same normal values, therefore, may be applied. Our technique for collecting capillary blood is given.

The range of concentration of serum protein in 150 normal persons was found to be 6.1 Gm. to 7.6 Gm. per 100 c.c., and the average 6.7 Gm. per 100 c.c. Values greater than 7.5 Gm. or less than 6.0 Gm. per 100 c.c. should be con-

sidered abnormal until proved otherwise. There is no difference between the normal values for total serum protein in males and females.

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Medical College of Virginia
Richmond, Va.

THE RELATION BETWEEN SPECIFIC GRAVITY OF BLOOD SERUM AND ITS PROTEIN CONCENTRATION*

Joseph M. Looney, M.D., Worcester, Mass.

THE application of the falling-drop method for the determination of specific gravity of blood plasma or serum proposed by Barbour and Hamilton[1] has stimulated the adoption of this technique in many hospitals for the estimation of serum proteins. Because the method for the determination of specific gravity is very precise and accurate, many investigators have been led to the belief that the estimation of serum protein from specific gravity figures by the formula of Moore and Van Slyke[2] is just as reliable. The formula

$$\text{Total Protein} = 343\ (\text{sp. gr.} - 1.007),$$

proposed by Moore and Van Slyke, was subsequently confirmed by Weech and his co-workers.[3, 4] These investigators found that the correlation coefficient between specific gravity and total protein had the remarkably high value of 0.994 for serum and 0.991 for plasma. They gave the following equations for the regression line between the two factors:

For Plasma:

$$P = 340.1\ (\text{sp. gr.} - 1.00687) \pm 0.103.$$

*From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester.
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For Serum:

$$P = 347.9 \text{ (sp. gr.} - 1.00726) \pm 0.076.$$

However, Zozaya,[5] in a study on the physicochemical characteristics of blood sera, found a correlation coefficient of only 0.28 ± 0.062 between total serum protein and specific gravity. He thought that his failure to obtain better results might have been due to the use of serum rather than plasma. Against this interpretation, however, is the finding of Nugent and Towle[6] that the specific gravity of synthetic solutions of serum albumin and serum globulin was a function of the total concentration of protein regardless of the distributions between the two substances. A recalculation of their data gives the following regression equation:

$$\text{Total Protein} = 366 \text{ (sp. gr.} - 1.0067).$$

For several years we have been accumulating data on the relationship between the protein content and the specific gravity of blood sera. We have not been able to confirm the findings of Moore and Van Slyke[2] or of Weech and his collaborators[3, 4] that there is a high correlation between specific gravity and serum protein, and that the percentage of protein in a given serum can be predicted with a high degree of accuracy.

METHODS

The material for our study has been drawn from three sources: first, from a group of 14 schizophrenic patients who were studied under basal conditions at four different times; second, from a group of 10 normal persons who were studied under basal conditions on two different days; and third, from a group of 12 patients undergoing diathermy therapy who were tested on two different days, before the treatment was begun, at the time that the temperature reached the maximum desired, and finally after the temperature was held at this point for three hours. The specific gravity was determined by the falling-drop method of Barbour,[1] using a solution of potassium chloride of density 1.0343 as the standard of comparison. The total protein was determined by the turbidimetric method of Looney.[7, 8] All determinations were made in duplicate.

The use of the turbidimetric method for the determination of the total protein in our hands has given results which statistically show less variation in duplicate determinations than the standard Kjeldahl method. In a series of 20 sera analyzed for total protein in duplicate by both methods, the following results were obtained: The Howe method gave a mean difference between duplicates of 0.052 mg., with a standard deviation of 0.051 mg. The Looney method gave the values of 0.046 and 0.034 mg. The mean of the differences between means of the duplicates of both series was 0.096 mg., and the standard deviation was 0.069 mg. It is evident, therefore, that there is no significant difference between the methods, and that the variance of the Looney method is somewhat lower than that of the Howe method. In this connection it may be pointed out that only the colorless drops of ghatti gum should be used in the preparation of solutions. If difficulty is experienced in obtaining a good quality of gum, then 2 per cent duponol can be used as a dispersive reagent.

RESULTS

In Fig. 1 the specific gravity of the sera of the patients on four different days was plotted against the total protein. The values for each person are connected by lines, and the order in which the tests were run is indicated by different symbols.

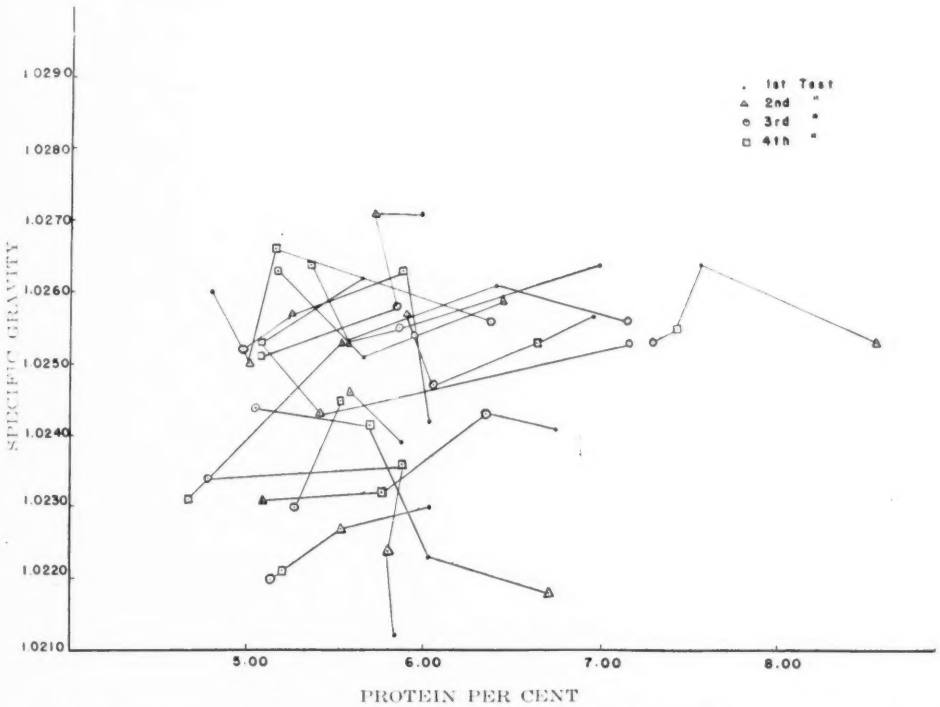

Fig. 1.—Correlation between specific gravity and serum protein in patients. Lines connect readings for individual patients on four different days.

It is evident from an examination of the graph that neither the specific gravity nor the total protein values for any person remain constant. It is also apparent that there is no consistent relationship between the specific gravity and the protein content of the blood serum. The group means for the specific gravity remained practically unchanged from day to day, the values being 1.0248, 1.0246, 1.0247, and 1.0248 for the successive days. The means for the total protein, however, showed considerable variability. The values in grams per 100 ml. of serum were 6.18, 5.87, 5.94, and 5.61. The correlation coefficients for each of these days also showed marked variation, as shown by the following values: 0.22, 0.04, 0.58, and 0.16. The coefficient of correlation for *all* the readings was 0.143.

The means of the total protein for the normal persons were more consistent, giving a value of 6.83 Gm. for the first day and 6.77 Gm. for the second. The specific gravity values gave the same mean 1.0269 for both days. Despite this constancy for the means, the coefficient of correlation was only 0.18. This lack of relationship is clearly shown in Fig. 2.

It was felt that a determination of the relationship between the two variables during diathermy would offer an ideal situation for a conclusive test of the

hypothesis. Marked hemoconcentration takes place during the period of fever despite the administration of copious amounts of water. The protein content of the blood would not be expected to change during the period of the experiment and should furnish a reliable measurement of the amount of fluid loss. If the specific gravity furnishes a reliable index of the total protein content of the blood, then a high degree of correlation should be found in each person tested.

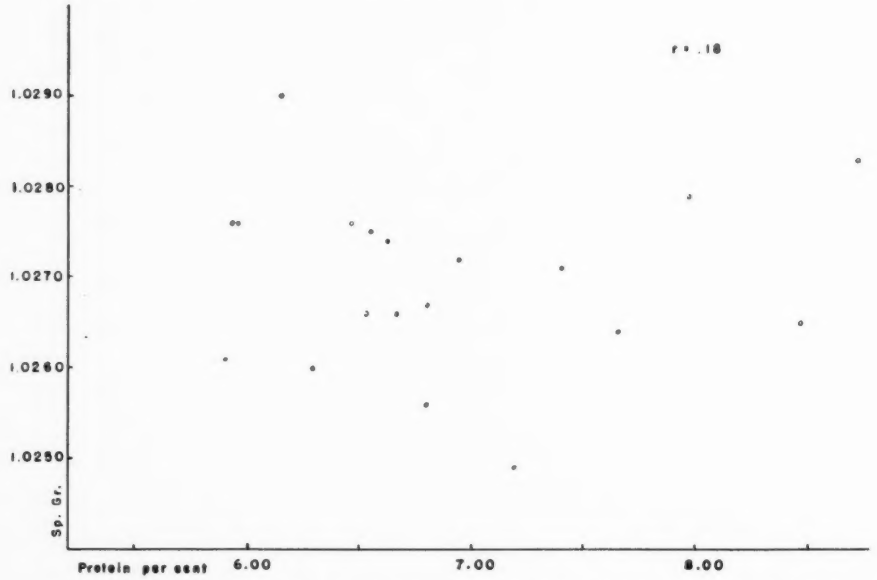

Fig. 2.—Correlation between specific gravity and serum protein in normal subjects.

The results of these experiments are plotted in Fig. 3. It will be noted that in some cases there is such a relationship and the three points can be connected by a line which is straight, or nearly so. In other instances, however, no such relationship occurs, and in one case a fall in specific gravity is associated with an increase in total protein. Moreover, in those cases in which a straight-line relationship was found between the specific gravity and total protein of the serum, the slopes of the lines were not the same. This fact makes the use of any single formula unsuited for the calculation of protein from specific gravity. An inspection of the figure will show that there is a wide spread in the values for specific gravity for the same value of total protein and an even greater spread of total protein for given values of specific gravity. The correlation coefficient for all the values is 0.563 and the regression equation is:

$$\text{Total Protein} = 315\ (\text{sp. gr.} - 1.0046).$$

The means for the total protein for the three periods were 6.60, 6.97, and 7.58. The corresponding values for the specific gravity were 1.0258, 1.0267, and 1.0283, and for the hematocrit readings were 40.01, 41.42, and 43.04. The correlation between the serum protein and the hematocrit reading was very low, since the coefficient was only 0.134 and there was no correlation between the specific gravity and the hematocrit reading.

DISCUSSION

The results of our investigations show that the specific gravity of blood serum is not a simple function of the concentration of protein and that the degree of error in calculating the protein value from the specific gravity in any individual case may be very large. This finding is not unexpected, since the amount of salts and fat in the serum should have considerable influence on the final density of the serum.

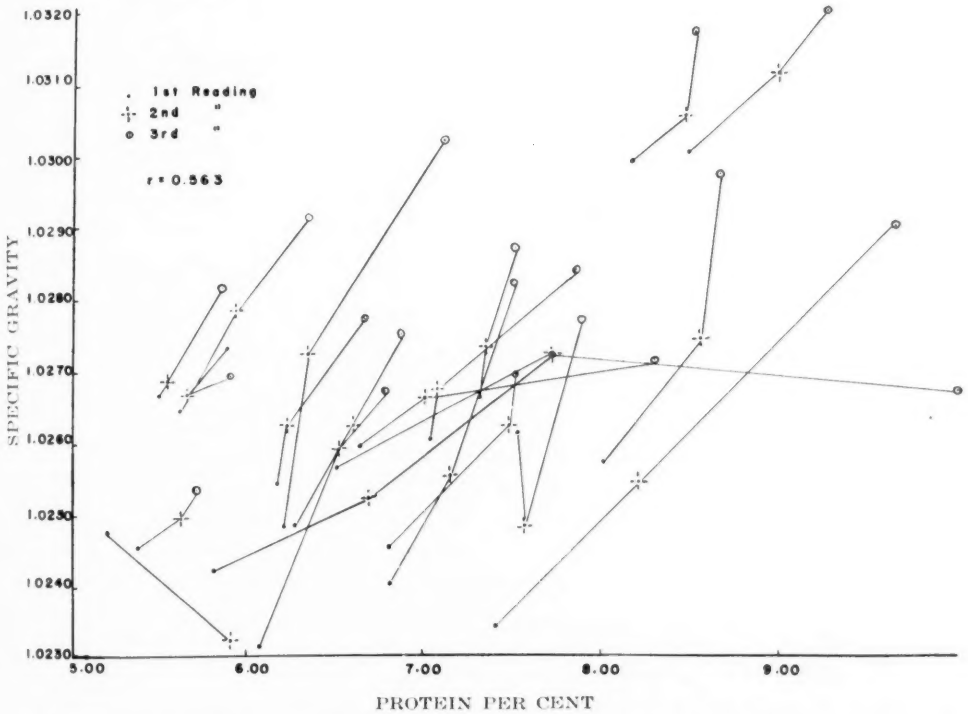

Fig. 3.—Correlation between specific gravity and serum protein during diathermy. Lines connect readings of individual patients.

Our experiments on the changes in protein concentration and specific gravity during the course of diathermic therapy indicate that the concentration of the serum proteins serves as a better indicator of fluid changes in the blood than either specific gravity or hematocrit value. This type of experiment should provide a crucial test of the relationship between density and protein concentration, since the total amount of circulating protein should remain virtually unchanged during the course of the experiment and the concentration should vary directly with the amount of fluid lost from the blood vessels. As a result the study of a smaller number of cases should provide as much information as that of a larger number of unrelated tests on a group of persons selected at random.

It is also evident that, although the amount of change in specific gravity produced by a given change in the concentration of serum protein may be fairly constant in a single person, it is not the same for all persons. The regression equation for a group would give a fair indication of the mean relationship

between the density and the protein concentration, but this would not hold for any single person.

These results are in agreement with the conclusions reached by Zozaya already quoted,[5] and also with the findings given by him in a later paper.[9] He gave figures showing that 12 samples of sera, each having a total protein concentration of 6.59 Gm. per 100 c.c., had specific gravities ranging from 1.0251 to 1.0277. The protein concentration calculated from these specific gravities by the formula of Moore and Van Slyke ranged from 6.21 to 7.10 Gm. per 100 c.c., or using the formula obtained from the data in this paper, from 6.46 to 7.28. In another group of 9 persons, all having a specific gravity of 1.0272 or 1.0273, the total protein values varied from 6.70 to 7.36 Gm. per 100 c.c. The calculated protein for these specific gravities would be 6.93 or 6.96 Gm. per 100 c.c. by Moore's formula and 7.12 or 7.15 Gm. per 100 c.c. by mine. The agreement between the mean protein value of 7.01 Gm. per 100 c.c. and those calculated from the foregoing formulas is good, but the variation from individual readings is rather high.

Similar findings were obtained by Moon and his collaborators[10] in a recent article on shock and hemorrhage. They found that during shock the specific gravity increased while the serum proteins decreased. In hemorrhage, however, both variables were diminished. In these experiments a decrease in the concentration of protein occurred during the period of hemoconcentration following shock, but this loss of protein was not indicated by the specific gravity which increased because of the action of other factors, such as the increased concentration of nonprotein nitrogen and salts except chlorides.

Barbour[11] has adopted the method for the estimation of serum albumin by determining the specific gravity of the supernatant solution from an equal mixture of serum and saturated ammonium sulfate and comparing it with a standard solution of ammonium sulfate. Although the standard deviation for the serum albumin estimated from the specific gravity differed from that obtained by Howe-Kjeldahl method by only 0.32 Gm. per 100 c.c., a study of the graph indicates that for any single case much larger differences may be found. Thus, for a serum albumin value of approximately 3.70 Gm. per 100 c.c., the specific gravity varies from 1.0245 to 1.0268, and for a specific gravity of 1.0245 the serum albumin varies from 2.30 to 3.70 Gm. per 100 c.c. While the method can be used for the determination of the mean value of the serum albumin for any given group within the accuracy indicated by the author, the application to any specific case is fraught with considerable danger since this may involve errors in such instances as great as 50 per cent.

A consideration of the data given in this paper, as well as those obtained from other authors, indicates that the correlation between serum proteins and specific gravity is not sufficiently close to permit accurate estimations of the former variable by a determination of the latter.

SUMMARY

The relationship between the specific gravity of serum and its protein concentration was determined repeatedly in a group of schizophrenic patients and also in a group of normal subjects. During diathermy the effect of prolonged

fever on the serum proteins and specific gravity was studied in a third group of paretic patients. The correlation between the two variables was found to be too low to permit accurate estimation of the serum proteins of persons from the specific gravity values.

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MEDICAL ILLUSTRATION

Editorial Comment

In consideration of the fact that physicians often speak during the showing of motion pictures that are neither titled nor recorded in sound, the following article should prove of considerable value as a guide to judging word spacing and timing between sequences. This article should also be of value to the scientific worker who plans to have sound recorded on a previously prepared film. In such a case exact timing is of utmost importance. It is logical to reason that with the development of sound recording devices and the obvious reduction in price that has rapidly taken place in the last few years, the sound motion picture for technical use, as in teaching and reporting at scientific meetings, will eventually replace silent films entirely.

It is worth bringing to the attention of the illustrator and physician the fact that sound can be dubbed in on practically any silent motion picture. Sound projectors and projectionists are usually supplied for the use of speakers at most of the larger meetings. The average sound projector costs about twice as much as a silent projector.

For those persons wishing to study the picture along with the text, prints of the picture may be obtained from the author at a nominal cost.

TEXT OF THE MOTION PICTURE: A LECTURE ON THE SPIROCHETES*

THEODOR ROSEBURY, D.D.S., NEW YORK, N. Y.

PHOTOMICROGRAPHS OF SOME OF THE PARASITIC AND PATHOGENIC SPIROCHETES AND SPIRILLA AS THEY APPEAR UNDER THE DARK-FIELD MICROSCOPE

(Magnification is uniform throughout, about 160 diameters on the film. An approximate scale in microns is given.)

Note.—The text is prepared to be read as the film is shown at standard silent speed, 16 frames per second. Numerals in parentheses, e.g., *(150)*, indicate the approximate rate of reading in words per minute. Other directions for reading are also given in italics in parentheses. Titles are given in capitals as section headings.

(Begin reading on the first dark-field scene) (150) The spirochetes are distinctive microorganisms, characterized as unicellular spiral filaments with a flexible body, motile without flagella. They are classified among the bacteria as a separate order, but they are sometimes considered intermediate between bacteria and protozoa. *(Wait 5 seconds)* We are going to examine the parasitic spirochetes individually and look into their relationships in disease. Before we do so let us make some general observations on the group as a whole.

*From the Department of Bacteriology, College of Physicians and Surgeons, and the School of Dental and Oral Surgery, Columbia University, New York City.

This film was shown at the Forty-Third General Meeting of the Society of American Bacteriologists, Baltimore, December 30, 1941. It was made to be used as a general lecture for medical and dental students. Copies of the film may be obtained at the cost of printing from the negative.

MORPHOLOGY AND CLASSIFICATION OF SPIROCHETES AND SPIRILLA

(On chart) (120) We recognize five genera of spirochetes, shown here diagrammatically with a group of spirilla for comparison. Let us look at them individually. *(Chart builds progressively; time to animation) (150)* The genus *Spirochaeta* properly includes only certain large spirochetes. Part of one is shown here. They occur as free-living forms in water and are not pathogenic. They have a spiral axial filament and regularly disposed granules. The genus *Cristispira* is likewise of no medical importance. These spirochetes usually occur as parasites in the alimentary canal of shellfish. They are large forms with a crest or crista, a flexible membrane wound spirally around the spiral organism. They have granules and a chambered structure. The genus *Saprospira*, also not important medically, consists of spirochetes free-living in foraminiferous sand. They resemble cristispiras, but have no crest.

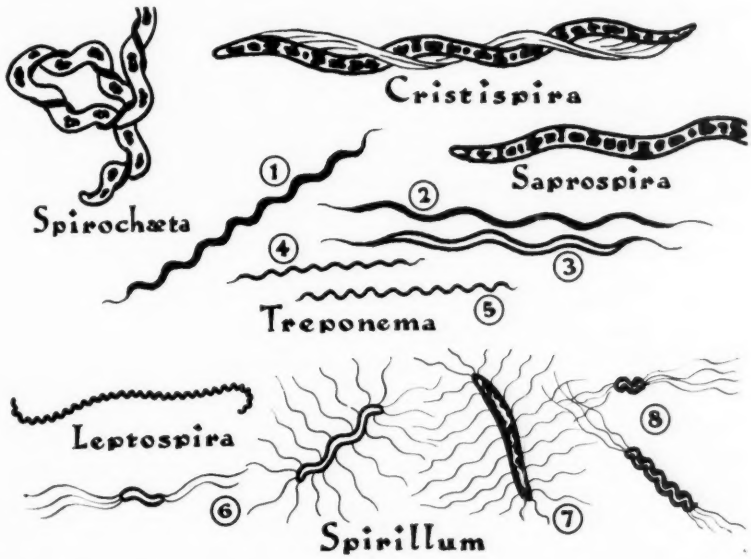

Fig. 1.—Chart of morphology and classification of spirochetes and spirilla. Numbers correspond with those given in the text.

(130) The generic name *Treponema* is used here to include most of the spirochetal species which are parasitic and pathogenic for man and animals. The treponemas usually show no details of internal structure. They are spiral filaments differing in width and in the number and shape of their spirals. These five forms, with their differences exaggerated in the drawings, are all represented in the photomicrographs: *(time to animation) 1*, the relapsing fever spirochete, intermediate in thickness and winding; *2* and *3*, the large spirochetes of mucous membranes, loosely wound, thin, or thick enough to show double contours; *4*, the small spirochete of mucous membranes, thin, tightly wound, with shallow spirals; and *5*, of which the spirochete of syphilis is the type, thin, tightly wound, with somewhat deeper spirals.

(130) The fifth and last genus of spirochetes is *Leptospira*, which includes both saprophytic and pathogenic species, all of which look somewhat like this.

MEDICAL ILLUSTRATION

Editorial Comment

In consideration of the fact that physicians often speak during the showing of motion pictures that are neither titled nor recorded in sound, the following article should prove of considerable value as a guide to judging word spacing and timing between sequences. This article should also be of value to the scientific worker who plans to have sound recorded on a previously prepared film. In such a case exact timing is of utmost importance. It is logical to reason that with the development of sound recording devices and the obvious reduction in price that has rapidly taken place in the last few years, the sound motion picture for technical use, as in teaching and reporting at scientific meetings, will eventually replace silent films entirely.

It is worth bringing to the attention of the illustrator and physician the fact that sound can be dubbed in on practically any silent motion picture. Sound projectors and projectionists are usually supplied for the use of speakers at most of the larger meetings. The average sound projector costs about twice as much as a silent projector.

For those persons wishing to study the picture along with the text, prints of the picture may be obtained from the author at a nominal cost.

TEXT OF THE MOTION PICTURE: A LECTURE ON THE SPIROCHETES*

THEODOR ROSEBURY, D.D.S., NEW YORK, N. Y.

PHOTOMICROGRAPHS OF SOME OF THE PARASITIC AND PATHOGENIC SPIROCHETES AND SPIRILLA AS THEY APPEAR UNDER THE DARK-FIELD MICROSCOPE

(Magnification is uniform throughout, about 160 diameters on the film. An approximate scale in microns is given.)

Note.—The text is prepared to be read as the film is shown at standard silent speed, 16 frames per second. Numerals in parentheses, e.g., *(150)*, indicate the approximate rate of reading in words per minute. Other directions for reading are also given in italics in parentheses. Titles are given in capitals as section headings.

(Begin reading on the first dark-field scene) (150) The spirochetes are distinctive microorganisms, characterized as unicellular spiral filaments with a flexible body, motile without flagella. They are classified among the bacteria as a separate order, but they are sometimes considered intermediate between bacteria and protozoa. *(Wait 5 seconds)* We are going to examine the parasitic spirochetes individually and look into their relationships in disease. Before we do so let us make some general observations on the group as a whole.

*From the Department of Bacteriology, College of Physicians and Surgeons, and the School of Dental and Oral Surgery, Columbia University, New York City.

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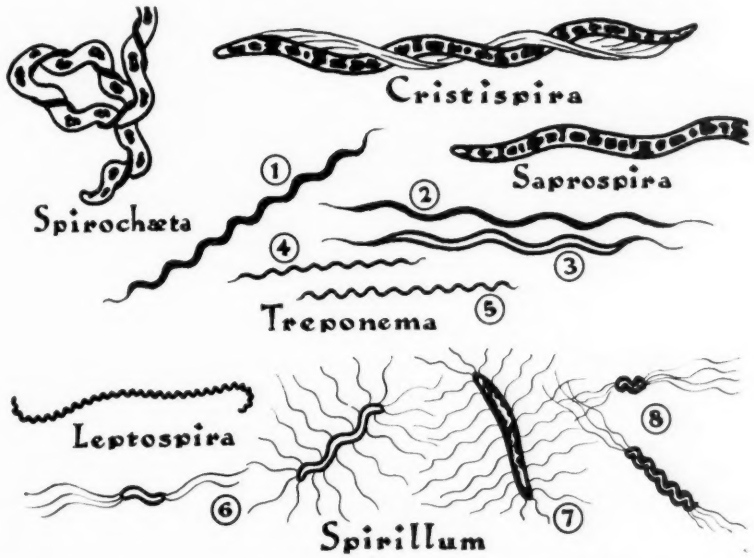

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(130) The fifth and last genus of spirochetes is *Leptospira*, which includes both saprophytic and pathogenic species, all of which look somewhat like this.

The leptospiras are much more closely wound than the treponemas, and characteristically one or both ends is bent to form a hook.

(130) The genus *Spirillum* belongs with the true bacteria, not with the spirochetes. The spirilla are flagellated and more or less rigid bacteria. The two forms shown at *6* are typical spirals or portions of a spiral, with flagella peritrichous or polar. Other spirilla, as shown at *7*, are curved rods with pointed ends. The rat-bite fever spirillum is *8*, short, thick, with rather closely set spirals, and with a tuft of flagella at each end. All these spirilla appear in the photomicrographs.

THE DARK-FIELD MICROSCOPE

(On diagram) (120) Dark-field examination at high magnification requires a substage reflecting condenser. This is a bicentric condenser, one of several kinds available, shown in place under a slide with the objective in position. The condenser has two reflecting surfaces, one concave and one convex. The whole condenser-slide-objective system must be made uniform in refractive index; therefore, oil is placed both above and below the slide.

PATHWAY OF LIGHT

(150) A narrow pencil of light passes from the mirror to fill the convex reflecting surface, which reflects it so as to fill the concave reflecting surface, and this concentrates the light to form a hollow cone. The cone is focused and centered in the plane of the specimen and in the optical axis. Incident light is stopped by a diaphragm in the objective, and only light reflected off particles in the field reaches the eye. The particles or microorganisms are thus illuminated like dust in a beam of sunlight entering a darkened room: they appear bright against a black background, and look larger or thicker than they really are because we see a halo of light around them.

(On preparation of the slide) (120) In preparing a slide for use under the dark field it is best to use a vaseline seal on the coverslip. Here vaseline is applied from a test tube with a wooden applicator. The seal prevents drying of the film and precludes drifts in the field which result from evaporation at the free edge of the film. *(Wait 5 seconds)* Both coverslip and slide must be scrupulously clean; bubbles should be avoided in the film since they reflect much more light than do microorganisms. The coverslip is pressed down to make a perfect seal.

(At microscope) (135) A small drop of oil is placed on the cover slip, and a larger drop on the under surface of the slide. A strong light source is used, such as an arc or a concentrated filament lamp. The light is focused through a collecting lens to a bright spot on the plane mirror, and the mirror is adjusted so that a concentrated beam of light fills the convex reflecting surface of the condenser. The objective diaphragm is stopped down, and the condenser is centered and focused so that the apex of the hollow cone of light falls in the optical axis and in the plane of the object. When this is done we see under low magnification *(bright spot appears)* a spot of light like this, which changes symmetrically to a ring of light as the condenser is racked either above or below the focus.

TREPONEMA RECURRENTIS FROM THE BLOOD OF AN INFECTED RAT

(Clipping tail) (120) A drop of blood from a rat infected with relapsing fever spirochetes is obtained by clipping the tail. The blood must be diluted for dark-field examination in order to separate the cells. The coverslip has previously been prepared with vaseline and a suitable diluting fluid, such as broth.

(On dark field) (150) Here we see red blood cells and *Treponema recurrentis* in a diluted rat blood film under dark field. *(Wait 2 seconds)* The discovery of these organisms, reported by Obermeier in 1873, brought out not only the first pathogenic spirochete but also one of the first microorganisms of any kind to be associated with disease in man.

(Wait 3 seconds) The motion of all spirochetes can be analyzed into three components: rotation around the long axis, flexion, or bending of the body, and translation or movement from place to place. The relative prominence of these components is a species characteristic, but varies considerably even in a single preparation. These spirochetes show active rotary movements and moderate flexion, but little translation. Others, taken from the same animal, which you will see a little later, show active movements of translation.

(Wait 3 seconds) Relapsing fever in man, as the name implies, is characterized by several sharp paroxysms of fever separated by irregular periods during which the symptoms are absent.

(Progressive movement begins) (150) There are two general types of relapsing fever. One, the so-called European type, is spread from man to man by the body louse, has occurred in severe epidemics, and often accompanies typhus fever. Both diseases are spread by lice, and, as the louse vector suggests, they are associated with poverty and crowding. Louse-borne relapsing fever is caused by *Treponema recurrentis*, a strain of which you see here. *(Wait 3 seconds)* The other type of relapsing fever is spread by ticks, which parasitize ground squirrels and other wild rodents. This type of the disease in man is sporadic rather than epidemic. It occurs in Africa and in South and Central America. Scattered cases have been reported in several parts of this country. Spirochetes have been recovered on numerous occasions from ticks taken in rodent burrows, especially in the western and southwestern states. The spirochetes of tick-borne relapsing fever are morphologically identical with the louse-borne strain you see here.

(Wait 3 seconds) (150) In relapsing fever in man, spirochetes occur in the blood during the onset phase of each paroxysm and diminish in numbers with its decline. They may be absent or difficult to find during the afebrile intervals. Diagnosis in man is best made by dark-field examination of a diluted blood film such as this, although staining methods can likewise be used on fixed films. The patient's blood should also be injected intraperitoneally into mice or rats, which are then examined for spirochetes in the blood after twenty-four to forty-eight hours. *(Elapsed time 12 minutes, 25 seconds)*

THE MOUTH SPIROCHETES AND ASSOCIATED BACTERIA IN EXUDATES FROM FUSOSPIROCHETAL ANGINA AND GINGIVITIS, AND FROM OTHER MOUTH DISEASES

(On patient) (150) Here we see the method of removing exudate from a case of Vincent's or fusospirochetal gingivitis. To provide enough material for

cultures and animal inoculation, as well as for smears and dark-field examination, *(making suspension)* a heavy suspension is made in 1 c.c. of broth.

(On dark-field) (135) Spirochetes similar to those of the mouth may also be found on the genitourinary and intestinal mucous membranes, and in a group of pathologic processes known collectively as the fusospirochetal infections. Several kinds of bacteria always accompany the spirochetes from any of these areas or disease processes. Among these bacteria *(fusiform bacilli appear)* are fusiform bacilli, of which one of several types is shown here; *(motile rod)* various motile bacteria, such as this small organism traveling in a circle, and filaments, as seen at the left; *(vibrio)* also vibrios, sometimes showing one or more polar flagella. Various cocci are always present. *(Spirillum)* Here is a spirillum, a characteristic mouth organism. Note the flagella. *(Motile spirillum)* And here is an actively motile spirillum of a different type.

THE MOUTH SPIROCHETES: TREPONEMA VINCENTI AND TREPONEMA BUCCALE

(On dark field) (150) These large loosely wound spirochetes of mucous membranes are easily distinguished from the smaller tightly wound forms, particularly when they are in active motion. Notice how these organisms *(spurts)* move forward in spurts, spinning rapidly and stretching their spirals almost taut as they spin, then stopping momentarily, relaxing, and reforming their spirals. This is a characteristic picture.

(Wait 2 seconds) (140) The names *Treponema vincenti* and *Treponema buccale* are applied to long loosely wound spirochetes which differ only in thickness. *Treponema vincenti* is the thin form, which appears as a single white line, or, as we say, is single contoured. *Treponema buccale* is thick enough so that light reflected off its opposite surfaces is resolved separately. It, therefore, appears as two white lines separated by a dark line; in other words, it shows double contours. Both spirochetes appear in these preparations, along with smaller spirochetes and various bacteria.

(150) These microorganisms can be demonstrated in small numbers in almost any mouth in which teeth are present, in material taken from the gingival crevice. They are not easy to find on really clean and healthy tissues, and never occur on such tissues profusely. Large quantities are indicative of a pathologic process.

(On heavy field) (150) Enormous numbers, like these, may, nevertheless, be found in a wide variety of pathologic conditions, and the number is not an index of severity. The condition may vary from a mild, localized inflammatory or ulcerative process all the way to fatal fusospirochetal gangrene, without any significant difference in the dark-field picture.

(Double contoured form) Note the double contours on the spirochete moving vertically. This is *Treponema buccale.*

(Wait 5 seconds) (150) Spirochetes found normally or in fusospirochetal lesions, in the anogenital region, have been given distinctive names, although they closely resemble the mouth spirochetes. For example, a genital spirochete called *Treponema refringens* looks like the large spirochetes of the mouth. It is important to distinguish these organisms from the spirochete of syphilis. This is

not hard to do if you remember that these larger nonspecific spirochetes are more loosely wound than the syphilis organism; these spirochetes rotate more actively and move forward much more rapidly when they move. Their spirals are more elastic, being lost and reformed during active rotation. It is worth remembering that *any* spirochete that shows double contours is *not* the spirochete of syphilis. This subject will be discussed more fully later.

(Wait 5 seconds) (150) Another differential feature of these large spirochetes is that they stain fairly well with aniline dyes, whereas the more tightly wound spirochetes, like *Treponema microdentium* and *Treponema pallidum*, do not. The large forms may, therefore, be prominent in stained films; the smaller spirochetes are best seen under dark field, although they can be stained by the Giemsa method or by silver impregnation. Films stained with aniline dyes often are used for the diagnosis of fusospirochetal infections, such as Vincent's angina, the diagnosis being based on the occurrence of these large loosely wound spirochetes and of large granular fusiform bacilli. This procedure may be useful in differential diagnosis, for example, in distinguishing between Vincent's angina and diphtheria; however, its positive value is doubtful because these organisms can be found in large numbers in conditions clinically distinct from Vincent's infection. In lung abscesses and other internal fusospirochetal infections, moreover, these larger organisms may be absent even though smaller spirochetes, small fusiform bacilli, and other bacteria can be demonstrated under the dark field. *(End of reel 1; elapsed time 19 minutes)*

TREPONEMA MICRODENTIUM AND OTHER SMALL MOUTH SPIROCHETES

(On dark field) (150) These tightly wound spirochetes of the mouth and other mucous membranes, commonly found along with the larger ones, are interesting for two general reasons. First, the small spirochete called *Treponema microdentium* is morphologically similar to *Treponema pallidum*, so that differentiation of the two forms is important in the microscopic diagnosis of syphilis. Second, the small spirochetes of the mouth seem to be essential members of the fusospirochetal flora, perhaps the most important of the group of microorganisms which appear to be responsible for Vincent's infection. We will consider both points in greater detail as we proceed.

MOUTH SPIROCHETES IN EXUDATE FROM INFECTED GUINEA PIGS

(On guinea pig) (150) Material containing the fusospirochetal flora, for instance, the exudate from Vincent's infection, pyorrhea or a lung abscess, when inoculated subcutaneously into the groin of a guinea pig, produces transmissible lesions which may take the form of a large necrotic abscess like this *(120)* or appear as a fatal diffuse gangrene of the subcutaneous tissues. In either case an abundant exudate, foul and usually fluid, can be aspirated from the lesion with a syringe, as shown here. *(Wait 5 seconds)* This abscess easily yielded about 5 c.c. of pus; some lesions contain 50 c.c. or even more. *(Wait 3 seconds)* The pus is frequently greenish, but may be cream colored. *(Wait 3 seconds)* In this instance it was bloody and dark in color. *(On tube)* It always has the foul odor characteristic of all fusospirochetal infections.

(On dark field) (120) Examined microscopically, the exudate from a guinea pig lesion usually shows fewer of the large spirochetes, particularly after several passages through guinea pigs. *Treponema microdentium* and other small spirochetes predominate, accompanied by bacteria of fewer varieties than are seen in the original human lesion material. Guinea pig passage eventually eliminates aerobic and gas-forming bacteria and many nonpathogenic anaerobes. The more limited flora that persists seems to represent the more pathogenic types: the smaller spirochetes, smaller nonmotile fusiform bacilli, and gram-negative rods of the Bacteroides group, as well as vibrios, streptococci, and other cocci. The whole flora in a stabilized guinea pig strain is anaerobic so that although the exudate contains myriads of living microorganisms, aerobic cultures made from it remain sterile.

(Pallidum-like spirochete appears) (150) Here is a form of *Treponema microdentium* which closely resembles *Treponema pallidum*. The two can be distinguished by their motility with some difficulty, as we shall see. When these spirochetes are not actively motile, as in this instance, the distinction becomes extremely difficult, if not impossible.

TREPONEMA MICRODENTIUM AND OTHER SMALL SPIROCHETES FROM CULTURES

(On plate) (150) *Treponema microdentium* and the other small spirochetes of the mouth can be cultivated without great difficulty. Here we see a culture of spirochetes in a small Petri plate being sampled with a capillary pipette by removing a portion of the medium containing spirochetes to a cover slip. The spirochetes grow in the depths of the medium, which is a solid nutrient agar containing ascitic fluid or serum ultrafiltrate and minced fresh sterile tissue, inoculated by a special technique and incubated in an anaerobic jar.

(On dark field) (120) *Treponema microdentium* is a small spirochete with regular closely wound coils. Some of the spirochetes seen here are small loosely wound forms, probably distinct from *Treponema microdentium* but as yet of uncertain nature. *(Wait 2 seconds)* The motility of *Treponema microdentium* is characterized by rapid rotation, often with active movements of translation. When movements of flexion are present, they frequently appear jerky or awkward, thus suggesting that the organism is relatively stiff.

(Wait 5 seconds) (120) These are the spirochetes which appear to be the contributing pathogens in fusospirochetal infections. They predominate in experimental lesions in guinea pigs and in internal fusospirochetal processes in man. Moreover, it has been reported that typical and transmissible fusospirochetal lesions could be produced in guinea pigs with combinations of pure cultures of four species: *Treponema microdentium*, a small fusiform bacillus, a vibrio, and a streptococcus, all anaerobes. Typical lesions could not be produced with any other or any lesser combination of species.

TREPONEMA PALLIDUM FROM THE TISSUES OF INFECTED RABBITS

(On rabbit) (160) The testicle of this rabbit, which is infected with *Treponema pallidum*, shows a large chancre. The chancre in rabbits, as in man, is an indurated swelling with surface ulceration that becomes encrusted. Material taken from the indurated tissue under the crust by aspiration with a capillary shows the characteristic spirochetes.

(On dark field) (120) Treponema pallidum, sometimes called *Spirochaeta pallida*, varies considerably in length, as do all spirochetes. Otherwise this organism is uniform in morphology, whether taken from the rabbit or from human lesions. Its coils are closely and regularly wound, often a little deeper than those of *Treponema microdentium*. *Treponema pallidum* is delicate; it never shows double contours under dark field. When it is not motile it resembles *Treponema microdentium* so closely that it is difficult, if not impossible, to tell them apart; but the two can be distinguished on the basis of motility. *(Wait 2 seconds)* Treponema microdentium, as we have seen, shows rapid rotation, frequently with active progressive movements. Its movements of flexion, when present at all, are rather awkward and give an impression of stiffness. *(Wait 2 seconds) Treponema pallidum* shows slower, more deliberate movements of rotation. Progressive movement is usually absent. The organism does not dart rapidly across the field. If it moves at all, it usually moves rather slowly and not far. *(Wait 2 seconds)* The spirochete of syphilis is characterized especially by its flexibility. It appears soft and its movements are, on the whole, graceful. It often looks like a streamer floating gently in the breeze. It has a certain dignity and elegance, befitting the aristocrat of spirochetes. *Treponema microdentium*, on the other hand, is the upstart, nervous and awkward in its movements. *Treponema pallidum* often exhibits a shimmering effect because of the combination of rather slow rotation with soft bending movements.

(Wait 3 seconds) (120) A demonstration of *Treponema pallidum* under the dark field is essential for the diagnosis of primary syphilis, and is also useful for rapid diagnosis in the secondary stage. In either instance, and particularly when the lesion is on or near a mucous membrane, contamination with nonspecific spirochetes should be avoided by expressing or aspirating fluid for dark-field examination from the depths of the lesion after carefully cleaning the surface. When the occurrence of *Treponema microdentium* or similar nonspecific organisms cannot be avoided and positive differentiation cannot be made, it is best to aspirate from a lymph node draining the area of the lesion.

(Sperm appears. Wait 5 seconds) (150) Treponema pallidum was not discovered until 1905, when Schaudinn and Hoffmann described it, along with *Treponema refringens*, in preparations made from syphilitic lesions. *Treponema pallidum* cannot be identified by ordinary methods. It cannot be cultivated by any of the methods used to grow bacteria; it does not stain with aniline dyes; and it is so delicate that it can hardly be seen at all in unstained preparations by direct illumination. Schaudinn and Hoffmann, nevertheless, discovered it without the use of dark-field illumination in fresh and in Giemsa-stained preparations.

(Wait 5 seconds) (150) The in vitro cultivation of virulent *Treponema pallidum* remains an unsolved problem. Noguchi and a few others have reported the cultivation of virulent spirochetes; other workers have either failed completely to grow them, or have obtained only avirulent spirochetes in culture which may not have been true pallidum at the beginning. Virulent *Treponema pallidum* is best grown and maintained for study in living tissues, commonly in the testicles of rabbits. In the rabbit testicle it produces a syphiloma or in-

durated swelling which may or may not ulcerate and form a chancre. This lesion usually appears in about three weeks, which is also the average incubation period of the chancre in man. The passage from rabbit to rabbit can be made in indefinite series. This is the Nichols strain of pallidum. It has been passed from rabbit to rabbit for about thirty years. In 1925 it was found pathogenic for man after accidental inoculation.

(Wait 5 seconds) (150) Experimental syphilis has been produced in chimpanzees and in some species of monkeys, as well as in rabbits. In the famous experiments of Metchnikoff and Roux on chimpanzees in 1903, two years before the discovery of the organism, all of twenty-two apes inoculated with human lesion material developed chancres and lymphadenitis, and some had secondary lesions. Most of the animals died of bronchopneumonia and no tertiary lesions developed. *(135)* Lower monkeys show only primary lesions. Rabbits are easier to infect than apes, and are the animals of choice for in vivo culture and for study of the experimental disease. In addition to the primary lesion they may develop generalized syphilis, with lesions that resemble both secondary and tertiary phenomena in man.

(Wait 5 seconds) (135) Mice, rats, and guinea pigs also can be infected with *Treponema pallidum*, but they usually show no lesions and spirochetes may not be demonstrable in their tissues. Their lymph nodes, spleen, and brain may, nevertheless, remain capable of infecting rabbits.

(On active movement) (120) The spirochetes shown in these preparations are as active as *Treponema pallidum* is ever likely to be. Notice that some of them show progressive movements, but that they move more slowly than *Treponema microdentium*, and much more slowly than the larger loosely wound spirochetes.

TREPONEMA PALLIDUM FROM LESIONS IN MAN

(On dark field) (120) These preparations were made from penile chancres. *(Wait 2 seconds)* Primary lesions in man often yield no more than one or two spirochetes per microscopic field; in fact, it may be necessary to search for the organisms and to make a diagnosis on seeing only one or two typical spirochetes. *(Wait 2 seconds)* Secondary lesions of the skin also show few spirochetes, as a rule, whereas secondary lesions on or near mucous membranes, mucous patches or condylomas, may show large numbers of pallidum. Skin lesions may, nevertheless, be more suitable for microscopic diagnosis, particularly by the inexperienced worker, because they are much less likely to be contaminated with nonspecific spirochetes. In any case, the surface of the lesion first should be cleaned carefully and fluid for microscopic examination expressed from the depths of the lesion.

(Wait 5 seconds) With a well-adjusted dark field, look carefully for double contours or for active progressive movements. Spirochetes showing either of these are not *Treponema pallidum*.

(Wait 2 seconds) (100) The spirochete of syphilis, as shown here, and this is worth repeating, is delicate, single contoured, and regularly wound with rather deep coils. It is flexible and graceful in its movements, rotates but does not spin rapidly, seldom moves from place to place, and if it does so generally moves rather slowly and not far.

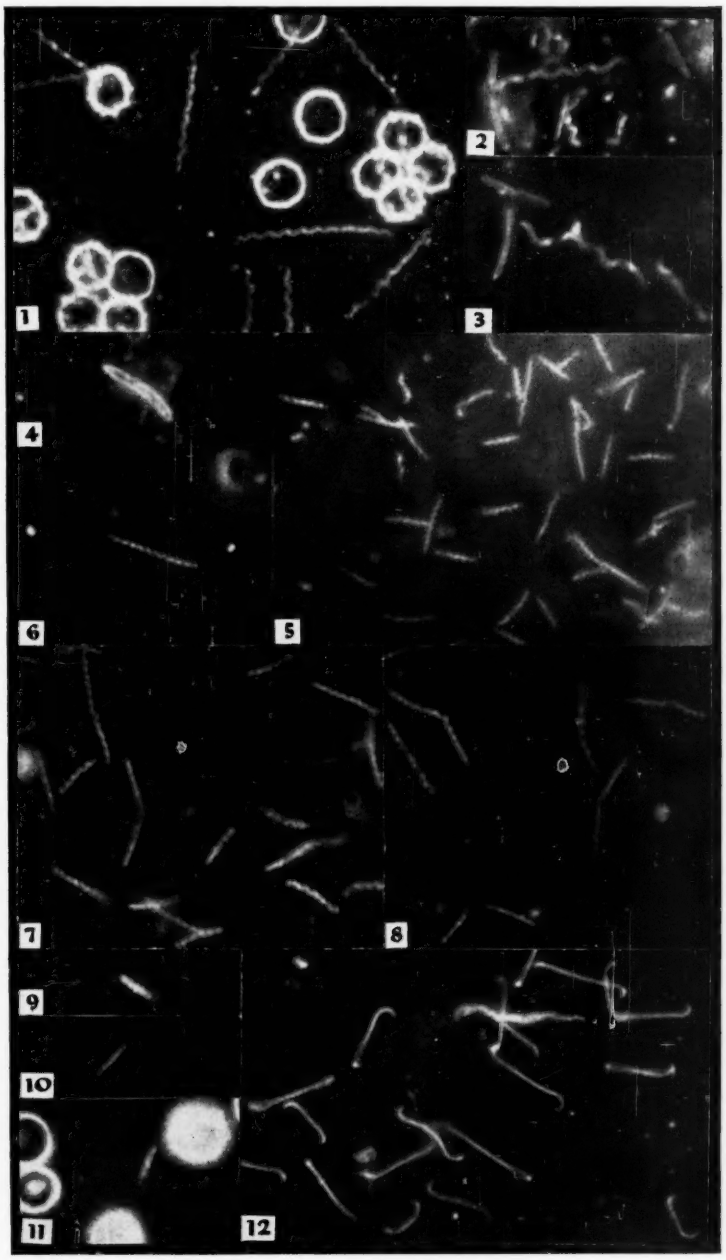

Fig. 2.—All figures are enlargements directly from 16 mm. negative frames. Magnification as reproduced is about 1,100 diameters. *1*, *T. recurrentis* (*novyi* strain) in rat blood; *2* and *3*, *T. buccale* and other microorganisms in gingival scrapings; *4*, A mouth spirillum *(Sp. sputigenum?)* in gingival scrapings, showing flagella; *5*, *T. microdentium*, probably mixed with other small mouth spirochetes, from a culture; *6*, *T. pallidum*, from a penile chancre in man; *7* and *8*, *T. pallidum* (Nichols strain) from rabbit testicular syphilomas; *9*, *10*, and *11*, *Spirillum minus* in mouse blood and peritoneal washings; *12*, *L. canicola* (strain D95) from a culture.

(Wait 5 seconds) (130) While different strains of pallidum are known to vary somewhat in thickness, even under uniform conditions, the organism sometimes appears exceptionally delicate, apparently through an artifact. This may happen if the exudate is kept for some time before microscopic examination, or if only a small amount of exudate is obtained and the film is exceptionally thin *(thin form appears)*, particularly if it is made without a vaseline seal. The spirochetes may then look so delicate as to be barely visible, even with the best dark-field equipment. Here you see a preparation of this kind.

TREPONEMA CUNICULI FROM AN INFECTED RABBIT

(On dark field) (120) *Treponema cuniculi*, which is shown here, is the causative agent of a naturally occurring venereal disease of rabbits, sometimes called rabbit syphilis, but not to be confused with experimental syphilis produced in rabbits by the inoculation of *Treponema pallidum*. *Treponema cuniculi* and another tissue spirochete, *Treponema pertenue*, the causative agent of yaws in man, are both morphologically indistinguishable from the spirochete of human syphilis. Like pallidum, they cannot be cultivated successfully and hence much remains to be learned about their biologic characteristics.

(Wait 3 seconds) (120) Yaws is a nonvenereal disease of man in tropical areas and occurs chiefly in children, with lesions confined mainly to the skin. The lesions of rabbit syphilis usually occur on the skin in the anogenital region, and are much smaller and softer than lesions of true syphilis. The syphilis and yaws spirochetes are immunologically related, but *Treponema cuniculi* is distinct. *(End of reel 2; elapsed time 37 minutes, 30 seconds.)*

LEPTOSPIRA BIFLEXA FROM A CULTURE

(On dark field) (130) This is *Leptospira biflexa*, a nonpathogenic spirochete found in water that contains decomposing organic matter. The several species of Leptospiras do not differ in morphology among themselves, but they are easily distinguished from the other spirochetes. They are much more closely wound than any of the treponemas, so that the individual spirals do not always show clearly; and they have the characteristic hook at one or both ends. Their movements are erratic and mercurial, they are the clowns among spirochetes. They rotate rapidly and often dart forward or to and fro; they may show quick lashing movements of flexion, or they may bend into crazy contorted figures.

LEPTOSPIRA ICTEROHAEMORRHAGIAE FROM A CULTURE

(On dark field) (120) *Leptospira icterohaemorrhagiae* is the spirochete of Weil's disease or spirochetal jaundice in man. The disease occurs throughout the world and has been reported in several parts of the United States. The organism was first isolated by Inada and Ido in 1915, and independently by Uhlenhuth and Fromme in the same year.

(Wait 5 seconds) (120) Weil's disease, in its more severe form, has a sudden onset with fever which may reach 105° F., and symptoms of acute intoxication. Jaundice may appear after about a week. The mortality may be as high as 50 per cent; but milder cases also occur, more chronic in character, often without jaundice.

(Wait 5 seconds) (120) Whereas all the treponemas appear to be more or less strictly anaerobic and can be cultivated with difficulty, if at all, the leptospiras are aerobic and grow readily in suitable media. They grow at room temperature in the upper centimeter or so of a column of serum or other organic fluid diluted with salt solution and containing a small amount of agar. Actively motile leptospiras like these are easily demonstrated in a loopful of culture removed from the upper portion of the medium and examined under dark field. Like the smaller treponemas, the leptospiras do not stain well with aniline dyes.

(Wait 4 seconds) (120) A characteristic feature of culture leptospiras in active motion is that they rotate so rapidly that their hooked ends look like closed eyelets, as shown by some of these organisms.

LEPTOSPIRA ICTEROHAEMORRHAGIAE FROM THE LIVER OF AN INFECTED GUINEA PIG

(On dark field) (150) Guinea pigs are exceptionally susceptible to Weil's disease; they usually die about seven to nine days after subcutaneous or intraperitoneal inoculation, and show intense jaundice of the skin and conjunctiva, with leptospiras demonstrable especially in the liver, kidneys, and adrenals.

(Wait 2 seconds) (150) In the diagnosis of Weil's disease a dark-field examination should be made of the blood early in the disease, or of the urine in the later stages, and 2 or 3 c.c. of blood or urine should be inoculated intraperitoneally into guinea pigs. At autopsy, or on about the ninth day after inoculation, dark-field examination of a suspension of the liver should show this picture. Young white mice may be used for inoculation, but older mice are resistant. Species of deer mice have also been used successfully.

(Wait 2 seconds) (150) As we shall see later, certain filaments which appear normally under dark field in blood or tissue suspensions may be mistaken for leptospiras. A true leptospira should show the characteristic hooked ends and at least some degree of rotary motion. These two characteristics justify a diagnosis of leptospira infection even though only a few organisms are found. The movements of flexion are unreliable, as is the apparent demonstration of spirals, since the filament artifacts bend in Brownian movement and may show irregularities that can be mistaken for spirals.

(Wait 2 seconds) (150) The leptospiras are sometimes called the water spirochetes, not only because *Leptospira biflexa* is found in water, but also because contaminated water is directly concerned in the transmission of Weil's disease. The infection has been common in Japan in wet mines in which the miners worked barefoot; elsewhere it has been associated with marshy or muddy soil, or with unsanitary bathing places; and cases have been reported both in Europe and in this country among sewer workers, fish handlers, and others who come into contact with wild rats or rat-infested water. Wild rats are frequently found infected with this organism and excrete leptospiras in their urine. Rats evidently serve as reservoirs of infection and disseminate it by the contamination of water. Transmission to man seems to occur mainly through the skin, the leptospiras being able to penetrate slight abrasions if not the intact skin itself.

TABLE I

DIFFERENTIAL DATA ON PARASITIC SPIROCHETES AND *Spirillum minus*

NAME OF ORGANISM	GENERAL CHARACTERISTICS	COILS*			TYPICAL ACTIVE MOTILITY			HABITAT OR SOURCE	ANIMALS USEFUL IN DIAGNOSIS, ETC.	NAME OF ORGANISM
		NUMBER	PITCH μ	OVER-ALL WIDTH μ	ROTATION	FLEXION	TRANSLATION			
T. recurrentis type	Intermediate in length, thickness, and winding; usually single contoured, may be double under critical illumination	6-10	2.5	1.5	Moderately active	Moderate	Moderately active; may be absent	Blood in relapsing fever during onset of paroxysm; rosette clumps during decline	Mice or rats; blood intraperitoneally, spirochetes in animal blood in 24 to 48 hours (stain with aniline dyes)	*T. recurrentis* type
T. vincenti type *(T. refringens)*	Long, thin; loose shallow coils; single contoured	3-6	4 or more	1-3†	Very active spinning, with near† loss of coils during progression, reformation on stopping	Not marked in fully active forms	Very active, intermittent spurts in one direction or reversing	Mouth, between gums and teeth; tonsillar crypts; anogenital mucocutaneous areas; fusospirochetal surface lesions	(Stain with aniline dyes)	*T. vincenti* type *(T. refringens)*
T. buccale type	Long, thick; loose shallow coils; double contoured									*T. buccale* type
T. microdentium type	Short, thin; tight shallow coils; single contoured	6-12	1.2	1 or less	Very active, coils unaltered	May be active, jerky, stiff, or absent	Actiye, darting; may reverse repeatedly	As above; also in internal fusospirochetal lesions (e.g., lung abscess)	Guinea pigs; exudate subcutaneously in groin; spirochetes in pus aspirated after 4 to 10 days	*T. microdentium* type

T. pallidum type	Medium length, thin to very thin, tight deep coils; always single contoured	8-12	1.2	about 1	Rather slow, deliberate	Active, soft, graceful	Usually absent; never darting	Lesions of primary and secondary syphilis; (*T. pertenue:* skin lesions of yaws; *T. cuniculi:* anogenital lesions of rabbit syphilis)	Rabbits (not for diagnosis); intratesticular; spirochetes in indurated tissue in about 3 weeks	*T. pallidum* type
Leptospiras (all species)	Short to long; thin to very thin, single contoured; very tightly wound; one or both ends hooked (easily cultivated, aerobic)	15 or more	about 0.6	about 0.5	Very active, spinning; hooks look like eyelets	Marked, with erratic lashing movements	Active; may be absent	*L. biflexa:* stagnant water; *L. icterohemorrhagiae:* wild rats and rat-infested water; blood (early), urine (late) in Weil's disease; *L. canicola:* dogs	Guinea pigs; blood or urine intraperitoneally; spirochetes in liver, kidneys, adrenals in about 9 days (also cultivation, serologic tests)	*Leptospiras* (all species)
Spirillum minus	Short, thick, but barely double contoured; coils appear closely set under dark field; bipolar flagella	2-5	1 or more	about 1	?	Slight or absent	Typical very rapid, darting	Mouse and rat blood, peritoneal fluid; bite wound and skin papules in rat-bite fever	Mice intraperitoneally (but spontaneous infection); guinea pigs; blood, exudate or excised tissue intraperitoneally or subcutaneously; spirilla in blood or peritoneal washings in 5 to 10 days	*Spirillum minus*

*These approximate measurements are based on the film. "Pitch" equals the distance between two coils as measured from crest to crest on one side. Length measurements are omitted because wide variation makes them of little differential value. The shortest *T. microdentium* may be 4μ, the longest *T. vincenti* about 25μ, but some specimens of *T. microdentium* are longer than some *T. vincenti*. Descriptions of motility apply to typical fully active forms; all species lose motility more or less gradually in slide cover slip preparations; dying forms may show erratic movements.

†Over-all width decreases during active rotation and progression because of stretching of spirals.

(Wait 2 seconds) (150) Weil's disease, unlike other spirochetal infections, confers lasting immunity. Agglutinins and lysins develop during the disease and persist for many years after recovery.

(Wait 2 seconds) (150) Noguchi, who made many outstanding contributions to our knowledge of spirochetes, particularly in the difficult problem of their cultivation, reported in 1919 that yellow fever is caused by a leptospira. The evidence for this view was gathered with Noguchi's usual thoroughness and it was widely accepted until 1928, when Stokes, Bauer, and Hudson showed that yellow fever is caused by a filtrable virus. Noguchi had apparently worked in 1919 with cases of Weil's disease mistakenly diagnosed as yellow fever. He later went to Africa to check his own findings. This time he worked with yellow fever and did not find leptospiras. While there Noguchi contracted yellow fever and died.

LEPTOSPIRA CANICOLA FROM A CULTURE

(On dark field) (160) *Leptospira canicola*, the leptospira of dogs, is morphologically identical with the other leptospiras. It can be distinguished serologically. It is less pathogenic for guinea pigs, but may infect both dogs and man. The prevalence of canicola infection in dogs is suggested by the finding of Meyer and his co-workers that agglutinins to *Leptospira canicola* occurred in a high percentage of normal dogs in urban areas of California. The *canicola* infection in man, contracted from dogs, is milder than Weil's disease, and may be so mild as to escape detection. Jaundice is usually absent; meningeal symptoms may be present. Leptospiras may be found in the spinal fluid or blood, later in the urine. By the seventh to the tenth day of illness or later, a diagnosis can be made by the agglutination of culture *Leptospira canicola* with the patient's serum.

SPIROCHETE-LIKE FILAMENTS: ARTIFACTS WHICH MAY BE MISTAKEN FOR LEPTOSPIRAS

(On dark field) (120) These filaments, seen here as delicate waving threads, sometimes attached to cells and sometimes free, are of no importance in themselves. They are not microorganisms. They are shown because they may resemble microorganisms, especially leptospiras, and thus give rise to errors in diagnosis. Although they are often homogeneous, they may show irregular swellings, clubbed ends, or beading. Occasionally they have a regular beaded structure, as shown here, which may give a false impression of spirals. Filaments occur frequently in dark-field preparations made from blood or tissue emulsions, particularly but not only when a hot loop has been used in preparing the slide. They may be extremely delicate and pale, or coarser and brighter, and vary from short rods to long waving threads. They have no independent motility, do not rotate or move progressively; but they may wave and bend actively in Brownian movement. The two features that best distinguish them from leptospiras are the absence of hooked ends and the lack of rotary movement. *(Elapsed time 48 minutes, 25 seconds)*

SPIRILLUM MINUS FROM THE BLOOD OF AN INFECTED MOUSE

(On dark field) (140) This organism, seen lying still among red blood cells, is a spirillum, not a spirochete. The difference, as we have seen, is that a

spirillum is a curved or spiral organism motile by means of flagella, while a spirochete moves, as far as we know, by means of the undulations of the body of the organism itself. Flagella have never been demonstrated on true spirochetes. They appear on these organisms, and you will see a tuft at each end in later sequences if you watch for them.

Spirillum minus appears under dark field as a short thick rodlike form, just barely double contoured, with rather closely set stiff spirals which are not always clearly visible.

(Wait 5 seconds) (140) This organism is generally regarded as the cause of rat-bite fever or Sodoku. Since its cultivation has not been successful, it has not been possible to fulfill Koch's postulates for *Spirillum minus* as the etiologic agent of rat-bite fever; however, inoculation experiments with unpurified animal blood or exudate have been successful in animals and in man. The relationship of this organism to rate-bite fever has been challenged in favor of a distinct bacterium called *Streptobacillus moniliformis*, which may also occur in the human lesions and in infected rats and mice, and which can be cultivated independently.

(Wait for flagellated form) Flagella can be seen on this organism and in several of these sequences. *(Wait for another flagellated form)* Note the tuft of flagella at each end *(and another)*, and here again.

SPIRILLUM MINUS IN PERITONEAL WASHINGS FROM AN INFECTED MOUSE

(On dark field) (120) When *Spirillum minus* moves, it darts rapidly across the field. You will see these darting movements in a little while.

(Wait 5 seconds) (130) Whether or not this organism is the cause of rat-bite fever, diagnosis can be made if it can be demonstrated under the dark field in exudate expressed from the bite wound or from the skin papules which accompany the paroxysms of fever. The bite wound usually heals during the incubation period, and later ulcerates and looks like an extragenital chancre. The demonstration of spirilla in the blood or peritoneal fluid of mice or rats after inoculation with the patient's blood is less reliable for diagnosis, since these animals may harbor the organism normally without showing symptoms. The subcutaneous inoculation of guinea pigs is probably a better procedure. Numerous instances of clinically typical rat-bite fever fail to yield spirilla. *Streptobacillus moniliformis* has been isolated from some of these. It seems likely that the streptobacillus is responsible for some cases, at least, of rat-bite fever. Recent findings suggest that there may be two types of the disease, clinically similar, although perhaps not identical, but caused by distinct organisms. *(Elapsed time 52 minutes, 25 seconds)*

(On apparatus) (120) The apparatus includes a rigid stand supporting the camera independently of the microscope, the two being joined without contact by a light-tight coupling. A prism side ocular permits visual examination of the field being photographed. In order to allow one-man operation, a solenoid mounted beside the camera release is actuated by a foot switch, as shown here.

The apparatus used to make this film was designed and built by Fred Rosebury, Department of Biochemistry, Columbia University.

Pure strains of spirochetes were provided through the courtesy of:

Venereal Disease Research Laboratory, Marine Hospital, Stapleton, S. I.: *T. pallidum* (Nichols strain).

Dr. Thomas B. Turner, Department of Bacteriology, School of Hygiene and Public Health, Johns Hopkins University: *T. recurrentis* (novyi strain); *T. cuniculi* (strain A); *L. icterohemorrhagiae* (human strain S. E.); and *Spirillum minus.*

Dr. Laura Florence, Department of Bacteriology, New York College of Medicine: *L. biflexa.*

Dr. Elberton J. Tiffany, Department of Bacteriology, Long Island College of Medicine: *L. canicola* (strain D95). *(Total elapsed time 54 minutes)*

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A SIMPLIFIED APPARATUS FOR DARK-FIELD CINEMICROGRAPHY*

FRED ROSEBURY AND THEODOR ROSEBURY
NEW YORK, N. Y.

THE apparatus described is that used for the preparation of the 16 mm. film "A Lecture on the Spirochetes."[1] Except for the microscope and camera, it comprises few and inexpensive items. It is designed to be operated by one person, and is arranged so that with a minimum of inconvenience records on motion-picture film may be made of specimens observed during routine visual microscopy. The apparatus is adaptable to bright-field, histologic or other microscopy, as well as to dark-field microscopy with the oil-immersion objective, although it was designed primarily for the purpose last mentioned.

The optical system used comprises a Leitz AT microscope with the fluorite oil-immersion objective, 1.95 mm., N.A. 1.32, fitted with an iris diaphragm, and the Leitz bicentric dark-field condenser, N.A. 1.20. The focusing prism side ocular (Fig. 1 *A*) is similar to that supplied as the optical portion of a Leitz Makam camera, the camera box and ground glass having been removed, and the lower element of a 10× ocular, convex surface upward, having been fastened immediately above the prism in the optical axis to shorten the focal length of the system. A celluloid shield, blackened and lined at the outer edge with black felt fitted to the eye, is attached to the end of the lateral member of the side ocular so that when the operator's eye is pressed against it extraneous light is excluded. No camera lens is used. The camera, which is supported independently of the microscope, as will be noted below, is joined to the optical system without contact by means of a light-tight brass baffle coupling (Fig. 2*H*), threaded to fit into the camera in place of a lens. The inner surface of the brass coupling must be dead black. A cylinder projecting upward from the top of the side ocular unit (Fig. 2*J*) fits into the coupling without contact, so as to make the light-tight baffle connection between the microscope and camera.

The whole assembly is fastened to a large heavy draftsman's board, which in turn stands on four No. 7 live rubber stoppers to absorb vibration (Fig. 3). The microscope is mounted on a steel plate which is bolted to the board and cushioned with live rubber washers ¼ inch thick. The holes in the plate through which the bolts pass are large enough so that the bolts do not touch the steel plate itself, but are held by rubber washers above and below the plate. The plate is fitted with three steel pegs which mate accurately in shallow holes in the base of the microscope.

The stand upon which the camera is mounted consists of a heavy cast iron base, a tubular steel post (Fig. 1*B*), and an adjustable bracket with ball crank (*C*), which are available as parts of a standard drill press. Firmly attached to the bracket is a carriage (*D*) bearing a tripod screw and its bushing, and with side guides made to support the camera with a snug fit. At the outer end of

*From the Departments of Biochemistry and Bacteriology, College of Physicians and Surgeons, and the School of Dental and Oral Surgery, Columbia University, New York City.
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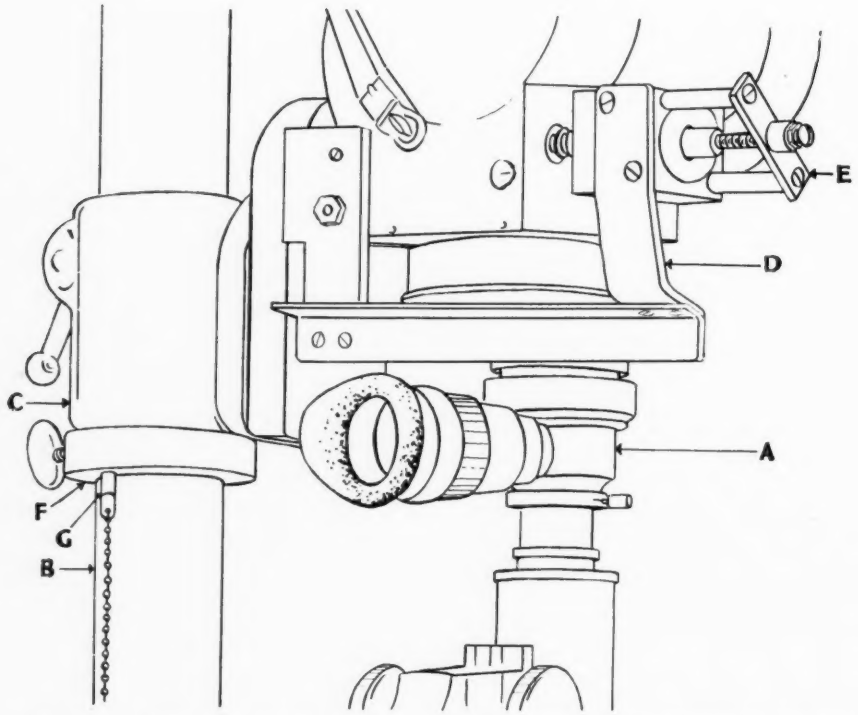

Fig. 1.—*A*, Prism side ocular; *B*, post supporting camera; *C*, adjustable bracket; *D*, camera carriage; *E*, electric solenoid camera release; *F*, collar; *G*, pin; *F* and *G* serve respectively as vertical and horizontal stops for the bracket and camera carriage.

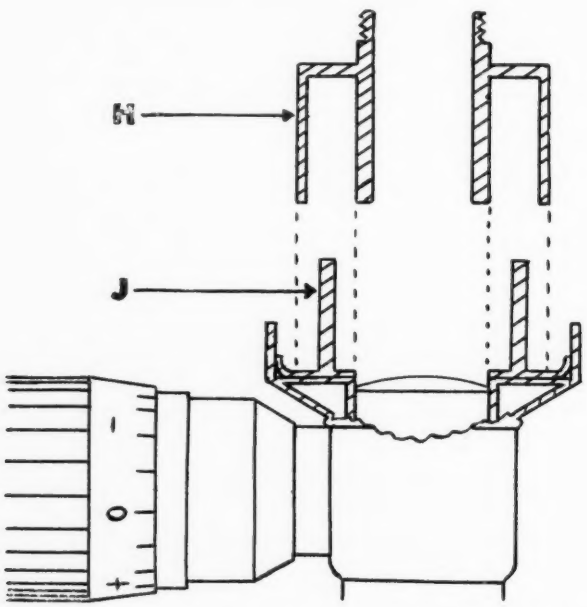

Fig. 2.—Detail of light-tight baffle joint between the camera and the prism side ocular, with the brass coupling (*H*) shown raised out of position above the cylinder (*J*).

the carriage is mounted an electric solenoid (*E*), the magnetic plunger of which is normally retained in a position on the outer side of the coil by means of an adjustable spring. When current is applied by means of a foot switch the plunger presses the release button of the camera. The end of the plunger carries a rubber bumper.

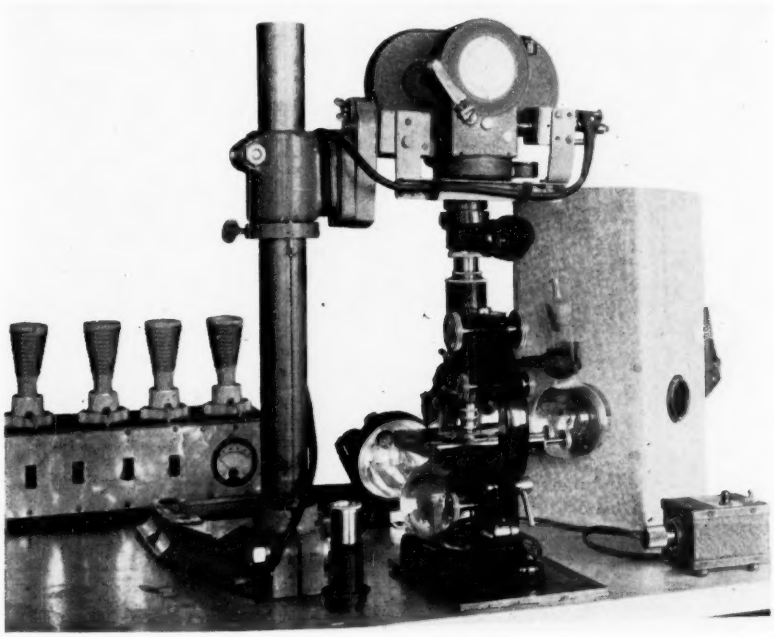

Fig. 3.—Photograph of the entire assembly.

A collar (Fig. 1*F*) is fastened to the post with a setscrew to serve as a vertical stop for the camera assembly and its bracket on the post. Horizontal locating is determined by means of a spring pin (*G*) in the collar, which fits accurately into a hole in the adjustable bracket. The spring pin is released by drawing down the chain attached to it. By this means the camera assembly can be lowered quickly into its proper position over the microscope and prism side ocular.

Two light sources are used, one for visual microscopy by bright or dark field and for bright-field photomicrography, the other for dark-field photomicrography. The former is a 6 volt automobile headlight lamp in a parabolic mirror reflector, operated on 110 volts A. C. through a variable voltage transformer (Bausch & Lomb). The stronger light source is a clockwork arc adapted to take National Projector carbons Nos. 698 or 722, ($\frac{7}{16}$″) and 168 ($\frac{5}{16}$″). The arc is operated on 110 volts D.C. through four 660 watt cone heater elements connected in series parallel with a switch in each parallel branch so that the arc current, which is read on an ammeter, may be conveniently adjusted in four steps. The resistances, switches, and ammeter are mounted on a transite and metal stand.

Both lamps are fastened in position on the draftsman's board so that their rays, condensed and cooled in each instance through a 250 c.c. flask of water,

are focused on the mirror of the microscope when the latter is in position on its steel plate. This provides a convenient arrangement for visual microscopy with the 6 volt lamp. When a specimen is seen which it is desired to photograph, the split prism with its ocular is substituted for the visual ocular of the microscope. The camera, with coupling attached, is swung and lowered into position with the aid of the collar and pin on the post and clamped tight, and the arc is turned on. With these operations, which require less than one minute, the apparatus is converted from a visual to a cinemicrographic assembly.

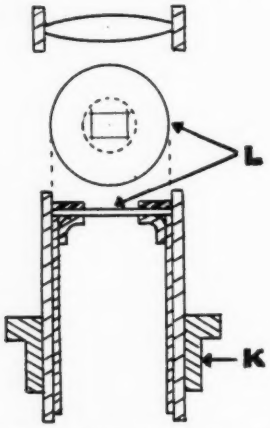

Fig. 4.—Focusing calibrator. *K*, Turned collar fitting into cylinder *J* in Fig. 2; *L*, cover slip ruled to 16 mm. frame size and placed in the focal plane.

The calibration of focus for the visual arm of the split prism ocular and the film was accomplished by means of the device shown in Fig. 4. A fiber cylinder 2 inches high was fitted with a turned wood collar (*K*) so as to set snugly into the baffle cylinder (Fig. 2*J*) of the split prism ocular. A clear circular cover slip (Fig. 4*L*) was then ruled with hydrofluoric acid to approximate a 16 mm. frame, and fastened in position in the cylinder at the level which would be occupied by the film when the camera was in place, as determined by measurement. A focusing magnifier could then be set into the cylinder from above so as to focus on the ruled cover slip. With this device the focus at the film plane could be matched with that in the visual arm of the split prism by varying the length of the latter. This calibration was checked by exposing and developing short lengths of film.

The film "A Lecture on the Spirochetes" was made chiefly on Agfa Superpan Supreme negative, which provided sufficient speed with maximum amperage of the arc (15 to 20 amperes). Photographs of specimens containing large numbers of cells, e.g., red blood cells, were in fact overexposed, although printable; and several sequences were made successfully with a slower film, DuPont 314 Pan. The film was developed, using Stineman tanks, in Agfa 17M at 18 C. for sixteen minutes with continuous agitation (gamma = 0.9). Under these conditions no objectionable grain appeared. Resolution on the negative seems remarkably good at the low magnification used (160 diameters), and, although slightly impaired in printing, it is considered satisfactory in the finished film.

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DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

DIPHTHERIA, Studies on the Antibacterial Property in, Huang, C. H. Am. J. Hyg. 35: 317, 1942.

Evidence pointing to the presence of antibacterial property in the diphtheria antitoxic serum is presented, based on the finding of the rapid disappearance of type D_5 organisms in the throats of patients during the first two weeks after treatment with antitoxic serum as compared with the slow disappearance of the organisms in the throats of patients infected by the other 8 types. Type D_5 organism is serologically the same as Park 8 strain which is universally used for the preparation of diphtheria antitoxin.

Specific agglutinins were found in the blood of diphtheria patients during the convalescent stage. In the acute stage such agglutinins were not found. A negative agglutination test with the blood of a diphtheria patient at the early stage of the disease and the appearance of specific agglutinins in the convalescent stage may, therefore, serve as a diagnostic method.

TARRY STOOL: Observations on the Oral Administration of Citrated Blood in Man. II. The Effect on the Stools. Schiff, L., Stevens, R. J., Shapiro, N., and Goodman, S. Am. J. M. Sc. 203: 409, 1942.

A tarry stool may result from the ingestion of 100 c.c. or more of citrated human blood. Following the intragastric administration of 1,000 to 2,000 c.c. of citrated human blood, gross blood may appear in the stools, and tarry stools may continue for five days and may number as many as 8. Positive chemical tests for occult blood in the stools may persist for as long as ten days after ingestion of 250 c.c. of citrated human blood or for twelve days after intragastric administration of 2,000 c.c.

The passage of a tarry stool does not necessarily indicate the occurrence of a severe hemorrhage into the digestive tract. The persistence of tarry stools or occult blood in the stools does not necessarily indicate the continuation of such hemorrhage.

CULTURES, A Note on the Preservation of Certain Bacteria Under Paraffin Oil. Simmons, R. T. M. J. Australia 1: 283, 1942.

Overnight slope cultures were completely covered with sterile liquid paraffin (heavy mineral oil; Parke, Davis Company) and stored in a dark cupboard at room temperature. The slopes should be moist, and they should be inoculated to the bottom of the slope so that some bacteria are suspended in the water of condensation which remains at the bottom of the slope under the paraffin. The paraffin was sterilized by dry heat at 160° C. in amounts of 15 milliliters heated for one hour, or in amounts of two liters heated for two hours. Autoclaving or the presence of moisture in the container caused turbidity. Paraffin from discarded cultures may be strained through gauze, sterilized and used again.

The top of the culture slope must be covered by the paraffin (preferably half an inch above) in order to prevent drying. A long, stiff, platinum loop was used to remove the growth from the bottom of the slope, where the organisms remained viable longer than at the top. After one year it was occasionally necessary to replace the paraffin with broth in order to obtain growth, but it was generally sufficient merely to transfer a loopful of organisms to broth.

PLASMA COAGULATION TIME, The Normal, Cheney, G. Am. J. M. Sc. 203: 325, 1942.

The plasma coagulation time is a satisfactory test for vitamin K deficiency, as previously noted.

The test has been carried out by a standardized technique in 340 normal persons and in 107 patients with nonhemorrhagic conditions.

The importance of a constant speed and time in centrifuging the blood has been emphasized.

The effect of changes in temperature upon the results of the test has been pointed out. Room temperature is satisfactory under ordinary conditions.

Standards have been set up for laboratory temperatures which may be encountered and for 37° C.

The constancy of the results obtained warrants the use of the plasma coagulation time as a simple laboratory test, provided the physical conditions of the procedure are adhered to closely.

The test of plasma coagulation time is routinely carried out as follows:

1. A clean venipuncture must be performed, obtaining the required amount of blood in a dry syringe.

2. The blood must be rapidly transferred to a test tube containing a weighed amount of dry potassium oxalate. A number of these tubes may be prepared at one time by adding 2 per cent potassium oxalate and evaporating the solution so that the tube will contain oxalate in the ratio of 10 mg. to 5 c.c. of blood. Centrifuge tubes are usually prepared with 5 mg. of oxalate for 2.5 c.c. of blood (0.25 c.c. of the 2 per cent solution per tube). The oxalate must be thoroughly mixed with the blood by inverting the corked tube ten or fifteen times.

3. The oxalated blood is centrifuged in a constant speed centrifuge at 3,500 r.p.m. for five minutes and the plasma is pipetted off.

4. Two-tenths cubic centimeter of plasma is pipetted into each of two small chemically clean test tubes, and 0.2 c.c. of 0.4 per cent calcium chloride is added to the first tube and 0.1 c.c. to the second. The tubes should be gently shaken five to ten times to secure thorough mixing. They need not be inverted.

A liter of the 0.4 per cent calcium solution may be made up at a time, using chemically pure calcium chloride.

5. The coagulation time of the plasma should be read in minutes. The end point is taken when the solution no longer flows when the test tube is held horizontal. The shortest time of coagulation for the two tubes is the plasma coagulation time.

6. The room temperature at which the test is carried out should be recorded. A variation between 23° and 26° causes no gross variation in the test.

7. The test should be completed within the first hour after the venipuncture.

CLOT RETRACTION TIME, A Method for Measuring, Hirschboeck, J. S., and Coffey, W. L. Arch. Path. 33: 380, 1942.

The equipment needed is a clean, dry syringe with needle and two dry test tubes, 1 cm. in diameter, one of which serves as a control. The tubes should be of soft glass and must be free of scratch marks. In addition they must be chemically clean. With the syringe and needle 4 c.c. of venous blood are removed, and 2 c.c. are placed in each of the test tubes. The tubes are closed with rubber stoppers, and the blood is shaken for a few seconds in order to create an equal amount of foam in each tube. The coagulation time is then determined by measuring the interval of time between the complete withdrawal of the blood from the vein into the syringe and the point at which the blood fails to flow when the tubes are completely inverted. The tubes are held in the same hand and are tilted every half minute in order to test for the end point. The end point of coagulation then becomes zero for the measurement of the retraction time. The tubes are placed in a rack kept at room temperature and are observed for the beginning of retraction.

The normal clot retraction time with this method is usually between twenty-five and thirty minutes. Some normal bloods may have a retraction time as short as twenty minutes; others, as long as forty-five minutes. The clot retraction time becomes shortened postoperatively, and if it is less than ten minutes, the patient may be considered a possible candidate for pulmonary embolism.

ANTITHROMBIN, Quantitative Studies on, Wilson, S. J. Arch. Int. Med. 69: 647, 1942.

A quantitative test has been devised for the determination of the antithrombin of serum and plasma. One unit of antithrombin is defined as that amount which will inactivate 1 unit of thrombin in four minutes at 28° C.

Normal human plasma or serum contains an average of 90 units of antithrombin per cubic centimeter, which is as much as a thousand times the amount revealed by many previous methods.

There is little or no difference in the antithrombic activity of serum and that of plasma.

During the actual process of blood coagulation only a small portion of the thrombin is adsorbed onto fibrin, the remainder being inactivated or neutralized by the normal antithrombic activity of the serum.

The hemorrhagic tendency in hypoprothrombinemia is not, therefore, fully explained on the basis of decreased prothrombin and the variations in the conversion rate of prothrombin to thrombin. The hemorrhagic diathesis usually occurs when the prothrombin unitage approximates or is lower than the antithrombin unitage.

The method follows:

The prothrombin-free fibrinogen was prepared by the method of Warner, Brinkhous, and Smith, all procedures being carried out at 5° C. and the materials stored at −35° C. The thrombin was prepared by the method of Seegers, Brinkhous, Smith, and Warner and standardized by either the pipette or the dropper method. The standardization tests should be repeated just before any critical observations are made.

The following method was devised to determine accurately fractions of 1 unit of thrombin: Fifteen drops of physiologic solution of sodium chloride were added to each of 9 test tubes. A series of 9 dilutions, containing 0.2 to 1.0 unit of thrombin in each drop, was then prepared, and portions of a single drop were added in sequence to the tubes of saline solution. To each test tube was then added 5 drops of fibrinogen (a total of 21 drops), and the appearance of the fibrin strands was timed with a stop watch. The following clotting times were observed:

Units of Thrombin	Clotting Time, Seconds
1.0	20
0.9	21.5
0.8	23.5
0.7	25.5
0.6	27.5
0.5	31
0.4	35.5
0.3	40.5
0.2	46.5

A curve to be used in the studies on quantitative antithrombic activity was then plotted. One unit of thrombin was used in the studies. If the figures for the thrombin are reversed, the curve will indicate the amount of thrombin inactivated or neutralized.

HEPATIC DEFICIENCY, The Value of the Hippuric Acid Test and Takata-Ara Reaction in the Investigation of, Henderson, M., and Splatt, B. M. J. Australia 1: 185, 1942.

A series of cases is reported of 313 hippuric acid excretion tests performed on 240 adult subjects.

The serum of 143 patients and of 50 normal subjects has been tested by the Takata-Ara reaction.

Quick's hippuric acid excretion test is both a reliable and a sensitive index of liver function, and is a valuable aid in estimating clinical progress, response to treatment, and prognosis.

It is simple to perform and is devoid of danger to the patient, but is not applicable to patients with poor renal function.

The Takata-Ara test is practically valueless as a test of liver function and is not a specific test for liver disease. It is, however, a useful confirmatory test for parenchymatous liver disease.

PREGNANCY, Colostrum Cutaneous Test for, Goldmann, L. M., Kessler, H. B., and Wilder, M. E. J. A. M. A. **119**: 130, 1942.

In 500 tests of cutaneous sensitivity to colostrum there were tested approximately 70 per cent correct reactions in all groups, both male and female.

There was apparently no greater or less efficiency of the test among endocrine cases.

In our hands the colostrum cutaneous test does not appear to offer a valuable diagnostic procedure for pregnancy.

AMINO-ACID, Plasma Levels in Health and in Measles, Scarlet Fever and Pneumonia, Farr, L. E., McCarthy, W. C., and Francis, T., Jr. Am. J. M. Sc. **203**: 668, 1942.

Thirty-one determinations of the plasma amino acid concentration of 30 normal, apparently healthy persons averaged 4.50 mg. per 100 c.c. by the ninhydrin–CO_2 method. The standard deviation was 0.46, and the observed range was 3.75 to 5.56 mg. per 100 c.c.

In patients with pneumococcus pneumonia the plasma amino acid nitrogen concentration was found to be low at the time of onset. With recovery from pneumonia the plasma amino acid concentration returned to the normal range. Patients admitted to the hospital after the fourth day of disease failed to show significant plasma hypo-amino-acidemia.

In patients with scarlet fever and measles the plasma amino acid nitrogen concentration was usually within the normal range. However, because of the difficulty of ascertaining the time of onset of these diseases, the data do not exclude the possibility of a plasma hypo-amino-acidemia during the pre-eruptive phase of these diseases.

PLASMA PROTHROMBIN Concentration, Clinico-Pathologic Correlation Between Hepatic Damage and, Sweet, N. J., Lucia, S. P., and Aggeler, P. M. Am. J. M. Sc. **203**: 665, 1942.

The prothrombin concentration was determined in 42 patients in whom the liver was examined at operation or at autopsy. Vitamin K was administered in adequate dosage to all those patients who had suffered from extrahepatic biliary obstruction. In general, the prothrombin concentration was normal when there was little or no destruction of the parenchymal tissue, and it was diminished when there was moderate or marked destruction of tissue. Exceptions to this rule are noted.

TUBERCLE BACILLI, Rapid Method for Concentration of, Oliver, J., and Reusser, T. R. Am. Rev. Tuberc. **45**: 450, 1942.

Five to 10 c.c. of sputum are mixed in a flask or widemouthed bottle, with approximately an equal amount of commercial chlorox, and shaken two or three times over a two-minute period and kept at room temperature for ten minutes. Then the mixture is centrifuged at 3,000 r.p.m. for ten minutes in a 15 c.c. conical tube. A creamy white sediment forms in the bottom of the test tube; the supernatant fluid is poured off and the tube is drained for two minutes. No neutralization is necessary. With an applicator a small drop of sediment is transferred to a slide. The slide dries at once and needs no fixing. Staining is done by the usual methods.